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Microvascular actions of insulin : studies on the interaction with angiotensin II and on the postprandial state

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MICROVASCULAR ACTIONS OF INSULIN

STUDIES ON THE INTERACTION WITH ANGIOTENSIN II AND ON THE POSTPRANDIAL STATE

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MICROVASCULAR ACTIONS OF INSULIN

STUDIES ON THE INTERACTION WITH ANGIOTENSIN II AND ON THE POSTPRANDIAL STATE

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General introduction and outline of the thesis

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Microvascular dysfunction in obesity: a potential mechanism in the pathogenesis of obesity-associated insulin resistance and hypertension.
Jonk AM, Houben AJHM, de Jong RT, Serné EH, Schaper NC, Stehouwer CDA
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INTRODUCTION

Microvascular dysfunction is common among the conventional cardiovascular risk factors, including obesity, insulin resistance, and hypertension.[1] Microvascular changes may contribute to insulin resistance trough impaired availability of glucose and insulin to muscle cells and to elevated blood pressure through an increase in peripheral vascular resistance.[1] Therefore, microvascular dysfunction might be a potential factor explaining the association between hypertension and insulin resistance as frequently seen in daily practice. Hypertension, insulin resistance, obesity, and dyslipidemia often cluster within individuals. This clustering of risk factors, referred to as the metabolic syndrome,[2] is associated with substantially increased overall and cardiovascular mortality.[3,4] Understanding the pathophysiology of this syndrome is important for the development of new therapeutic strategies. This thesis focuses on a role of microvascular dysfunction as a potential factor explaining the clustering of these risk factors.

Microcirculation – structure and function

The microcirculation is widely taken to encompass vessels <150 μm in diameter, including arterioles, capillaries and venules.[5]

The major function of the microcirculation is to regulate tissue perfusion to ensure adequate delivery of nutrients, oxygen, hormones, and removal of waste products; to provide exchange surface area between the plasma compartment and tissue interstitium; to avoid large fluctuations in hydrostatic pressure; and to regulate peripheral vascular resistance.[5] Adequate microvascular perfusion is therefore essential for the regulation of blood pressure and tissue metabolism.[5] In muscle and skin, capillary perfusion is regulated by terminal arterioles.[6] Terminal arterioles feed capillary units formed by about 12-20 capillaries each and finely regulate the qualitative and quantitative aspects of blood flow through these units. Due to periodic changes in terminal arteriolar diameter (i.e. vasomotion), not all capillaries are perfused at any one time; i.e. depending on whether their parent arteriole is "open" or not.[6] Therefore, under resting conditions, a part of the microvascular network of most organs remains closed, constituting a flow reserve that is used to adapt to increased metabolic needs under nonresting conditions.[7] To match microvascular perfusion to metabolic demand, acute regulation of the arteriolar diameter is achieved by the release of vasodilator (e.g. nitric oxide (NO), endothelium-derived hyperpolarization factor (EDHF) and prostaglandins (PGI2/PGE2)) and vasoconstrictor factors (e.g. endothelin-1 (ET-1)) by the endothelium.[8] Endothelial cells form the inner lining of the microvasculature and are in direct contact with blood and serum factors, like fatty acids, nutrients and hormones. The endothelial cells react to these factors through signal transduction cascades that regulate the production of vasoactive substances. Accordingly, vasodilation of precapillary arterioles leads to an increase in the total "open"

time of capillary networks to expand exchange surface (i.e. capillary recruitment), whereas vasoconstriction leads to the opposite.[6] Insulin is one of the many physiological factors involved in the vasoregulatory function, as it is able to induce NO and ET-1 release by the endothelium.[9,10]

Insulin: a vasoregulatory hormone

Insulin resistance is typically defined as decreased sensitivity and/or responsiveness to metabolic actions of insulin that promote glucose disposal. A major action of insulin in muscle involves translocation of the insulin-responsive glucose transporter (GLUT-4) to the plasma membrane and activation of downstream pathways of glucose metabolism.[9] However, before insulin interacts with the receptor on the plasma membrane, insulin and glucose first must be delivered to tissue interstitium in an appropriate amount and time course. In the past decades, it has become increasingly evident that insulin itself has an important vasoregulatory function.[9]

In the 1990s Baron et al. and others were the first to report insulin's ability to vasodilate resistance vessels to increase total blood flow in skeletal muscle.[11-14] Mainly because the ability of insulin to dilate skeletal muscle vasculature is impaired in a wide range of insulin-resistant states (e.g. hypertension, obesity, type 2 diabetes), it has been hypothesized that vasodilatory and metabolic actions of insulin are functionally coupled.[9,15] However, it remains controversial whether insulin's action to increase total muscle blood flow contributes to insulin-mediated glucose uptake, since insulin-mediated changes in total blood flow appear to have time kinetics and a dose-dependence on insulin different from those for the effect on glucose uptake.[16,17] Moreover, a number of vasodilators were shown to increase total muscle blood flow during hyperinsulinemia without any changes in glucose uptake.[13] These apparent contradictions in the data relating to insulin's effects on muscle blood flow and metabolism resulted in a shift of focus from insulin's macrovascular actions to insulin's microvascular actions. Clark and colleagues have introduced the concept that distribution of blood flow within the microcirculation, independent of changes in total muscle flow, may be important for insulin-mediated glucose metabolism.[14,18,19] By elegant studies in rats, applying different techniques to measure capillary recruitment (1-methylxanthine (1-MX) metabolism), microvascular volume (contrast-enhanced ultrasound), and microvascular perfusion (laser Doppler flowmetry), they demonstrated that insulin mediates changes in muscle microvascular perfusion consistent with capillary recruitment.[17,20-22] Insulin-induced capillary recruitment is induced by vasodilation of terminal arterioles connected to nutritive capillary networks and the resultant redistribution of blood flow from putatively non-nutritive (transit and/or connective tissue) to nutritive (capillaries) vessels.[15,23,24] This capillary recruitment is associated with changes in muscle glucose uptake independently of changes in total blood flow, requires lower insulin concentrations than necessary for

changes in total blood flow, and approximates the time course for insulin-mediated glucose uptake in skeletal muscle (i.e. 5-10 min).[10,17,25] Specific inhibition of insulin-induced capillary recruitment by inhibitors of endothelial nitric oxide synthase (eNOS) or infusion of the peripheral vasoconstrictor α-methylserotonin (αMT) have shown that this action accounts for ~40% of insulin-stimulated muscle glucose uptake.[22,25,26]

In humans, muscle microvascular responses to physiological hyperinsulinemia have been difficult to assess due to the lack of non-invasive techniques. Nevertheless, the cutaneous circulation has emerged as a valuable model of the microcirculation and may be considered as representative of the microcirculation in skeletal muscle.[27] Using intravital capillary microscopy and laser Doppler flowmetry a number of studies have reported a stimulatory effect of insulin on microvascular perfusion and vasomotion of human skin.[28-33] In 2001, contrast-enhanced ultrasound was introduced as a new technique to measure changes in muscle microvascular volume in response to hyperinsulinemia non-invasively in humans.[34] Subsequently, capillary recruitment in response to a hyperinsulinemic clamp and mixed meal feeding has been reported in the forearm muscle of healthy lean individuals.[35-39] Other methods, like laser Doppler flowmetry, have also been used to determine changes in microvascular perfusion in human muscle.[28] Importantly, in human studies, a strong association between insulin-mediated capillary recruitment, both in skin and skeletal muscle, and insulin-mediated glucose uptake was found.[29,30,33,35,38]

Vascular insulin signaling

Insulin's vasodilatory actions are mediated by the release of NO from the endothelium.[22,25,26] Insulin acts on the endothelium to activate the insulin receptor substrates (IRS)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB or Akt)/eNOS pathway,[9,40] leading to increased NO production and the dilatation of peripheral resistance arteries and terminal arterioles. In addition to this NO-dependent vasodilator effect, insulin also causes vasoconstriction, mainly through activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase-1/2 (ERK1/2) pathway and the production of the vasoconstrictor peptide ET-1.[40,41] Thus insulin has opposing endothelial-derived vasodilator and vasoconstrictor effects. In the healthy, insulin-sensitive state, insulin fine-tunes vascular tone and tissue perfusion by balancing its signals through these two signaling pathways. Noticeably, the insulin signaling pathways in the endothelium share striking similarities to the metabolic insulin signaling pathways in muscle cells.[9,42] This further indicates a functional, synergistic, coupling between the metabolic and vascular effects of insulin under healthy conditions and a synergistic coupling of metabolic insulin resistance and endothelial dysfunction under pathological conditions.[43]

Microvascular dysfunction: linking insulin resistance and hypertension

It has been established that both insulin resistance and hypertension are characterized by microvascular dysfunction.[1] Microvascular changes occur very early, even before the clinical manifestation of these conditions, and may thus be important in their pathogenesis and progression.[44-47] Importantly, by affecting both peripheral resistance and glucose metabolism, microvascular changes that result from hypertension could also predispose to insulin resistance and vice versa (Figure 1.1). In the following subsections the role for microvascular dysfunction, in particular microvascular insulin resistance, in the association between insulin resistance and hypertension will be discussed.

Insulin resistance

Most studies examining microvascular function in the insulin-resistant state have been performed in obese individuals. These studies have demonstrated that insulin-resistant individuals are characterized by several impairments in the microvasculature. The presence of endothelial dysfunction has been established by blunted NO-mediated vasodilator responses in skin and resistance arterioles to classic endothelium-dependent vasodilators[29,48-53] and impaired capillary recruitment to reactive hyperemia.[29,54] At the same time, insulin-resistant patients have elevated plasma ET-1 levels.[55] In addition, effects of insulin on NO and ET-1 availability are altered. A key feature of insulin resistance is that it is characterized by specific impairment in PI3K-dependent signaling pathways, whereas its signaling through the MAPK pathways remains intact.[56-58] Indeed, several studies have demonstrated impaired vasodilation and capillary recruitment in response to insulin in skin and skeletal muscle of insulin-resistant individuals.[29,30,35-37,59,60] This has important pathophysiological implications because metabolic insulin resistance is usually accompanied by compensatory hyperinsulinemia to maintain euglycemia. In the vasculature, hyperinsulinemia will stimulate unaffected MAPK–dependent pathways, leading to decreased production of NO and increased secretion of ET-1.[40,41,57,61,62] As a consequence, vasoconstriction of resistance arteries and terminal arterioles occurs, leading to disturbed regulation of muscle perfusion, glucose uptake and blood pressure.[5]

The cause of metabolic and vascular insulin resistance is still not resolved and how they are closely related is not fully understood. Specifically the temporal relationship between the two is intriguing. A widely held notion is that metabolic dysregulation (i.e. loss of muscle cell insulin signaling) is the primary abnormality and that endothelial dysfunction merely represents the impact of hyperglycemia and other metabolic consequence of insulin resistance.[63,64] According to this notion, inappropriate fat accumulation in muscle and liver cells or the release of inflammatory cytokines by fat cells might directly affect muscle cell insulin signaling.[63,65-67] However, most published results of insulin-signaling defects in skeletal muscle have been drawn from individuals with long-standing type 2

diabetes.[68] This clearly suggests that, once type 2 diabetes has become entrenched, major defects develop in the insulin-signaling pathway of the muscle cells. However, this does not necessarily identify the initial causes of the insulin resistance.[67]

An alternative concept is that changes in the microvasculature precede impairments in muscle cells. Thus, an early microvascular dysfunction, including loss of insulin-mediated capillary recruitment, can cause extensive periods of poor delivery of insulin, glucose, and other nutrients, and consequently decreased insulin-mediated glucose disposal, the hallmark of insulin resistance in muscle[15] (Figure 1.1). The resulting hyperglycemia will then contribute to the downstream defects in insulin action in muscle. Experiments where insulin-mediated capillary recruitment was blocked acutely and subsequent impairments in glucose disposal developed support the notion that loss of capillary recruitment can impact on insulin resistance prior to muscle cell insulin resistance.[22,25,26,69] Further evidence for the concept that the loss of microvascular function is a primary event is given by a study examining the temporal pattern of diet-induced insulin resistance in both vasculature and skeletal muscle which demonstrated that endothelial dysfunction developed well before impaired insulin activation of PI3K in skeletal muscle and liver.[70] It is also substantiated by a study in the Zucker diabetic fatty rat in which diminished NO signaling in skeletal muscle arterioles preceded the development of diabetes and hypertension.[71] In addition, there is considerable evidence that endothelial dysfunction in many vascular beds is a predictor of the onset of insulin resistance and diabetes,[72-75] underscored by studies showing endothelial dysfunction in mildly overweight, normoglycemic individuals with a strong family history of type 2 diabetes mellitus.[45]

A third concept is that insulin resistance in obesity is in essence an adaptive phenomenon and that impairments in insulin signaling in vessels and muscle develop in parallel, without one causing the other (i.e. insulin signaling in muscle and endothelium is very similar). Endothelial dysfunction and metabolic dysregulation then act mutually on each other.

Taken together, impaired delivery of glucose and insulin due to impaired vasodilation of terminal arterioles may be the key issue in the development of obesity-related insulin resistance. Importantly, impaired (insulin-mediated) microvascular vasoreactivity might also contribute to the development of hypertension and account in part for the epidemiological relationship between insulin-resistance and hypertension.[3,76] Specifically, the imbalance in endothelium-dependent vasoregulatory functions towards vasoconstriction, as seen in the insulin-resistant state,[40,41,57] elevates total peripheral vascular resistance and contributes to the development of hypertension.[1] Indeed, previous investigations have demonstrated that impairments of PI3K-dependent signaling of insulin in the endothelium of small arteries and arterioles directly contribute to elevated peripheral vascular resistance and hypertension.[77] Furthermore, a recent study which investigated whether vascular insulin

resistance acts as an early etiologic factor for the development of hypertension has demonstrated vascular insulin resistance and impairments of PI3K signaling in vascular endothelium of young hypertension-prone rats with normal blood pressure, implicating a causative role for vascular insulin resistance in hypertension.[78]

Hypertension

Hypertension per se is characterized by functional as well as structural changes in the microcirculation,[1] including impaired vasoregulation,[79] increases in wall-to-lumen ratio of small arteries,[1,80] and structural and functional capillary rarefaction.[5,76,81,82] The presence of endothelial dysfunction in hypertension has been established by blunted vasodilator responses and capillary recruitment to classic endothelium-dependent vasodilators (e.g. acetylcholine) and mechanical stimulation (shear stress).[76,83] Recent studies also demonstrated impaired NO-dependent vasodilation in response to insulin[77,78,84] in different animal models of hypertension. In addition to the decrease in NO availability, hypertension is characterized by augmented vasoconstrictor activity of endothelium-derived contracting factors ET-1 and angiotensin II (AngII).[79,85]

Whereas it has been known for many years that these microvascular alterations can be secondary to sustained elevation of blood pressure,[86,87] there is also evidence that microvascular changes can be a cause rather than a consequence of hypertension.[47,88,89] Microvascular abnormalities occur early during development of hypertension in spontaneously hypertensive rats[77,90] and prevention of oxidative stress by antioxidant treatment not only prevents rarefaction[90] but also prevents the age-related development of hypertension.[91] Furthermore, capillary rarefaction, similar to the magnitude seen in patients with established hypertension, can already be demonstrated in individuals with borderline hypertension[88] and in individuals with a familial predisposition to hypertension, even if they themselves are normotensive.[44,47] Thus, it seems likely that microvascular abnormalities can both result from and contribute to hypertension, and a "vicious cycle" may exist in which the microcirculation maintains or even amplifies an initial increase in blood pressure. However, microvascular dysfunction (including vascular insulin resistance) due to hypertension may also directly reduce the access of insulin and glucose to skeletal muscle, resulting in reduced insulin sensitivity (Figure 1.1).

In conclusion

In conclusion, although a possible mutual interaction between metabolic and vascular actions exists, there are several arguments for a causal role of microvascular insulin resistance and dysfunction in metabolic insulin resistance and hypertension. Subsequently, the hyperglycemia and hyperinsulinemia that evolve with metabolic insulin resistance can further impair endothelial function[92,93] and consequently impair glucose disposal

and increase blood pressure. In addition, hyperinsulinemia can induce blood pressure elevation by activating the sympathetic nervous system and the renin-angiotensin system (RAS), resulting in sodium retention and volume expansion, and endothelial and renal dysfunction.[94,95] Thus it is possible to envisage a vicious circle of progressive microvascular dysfunction that contributes to and is exacerbated by worsening metabolic insulin resistance and hypertension (Figure 1.1).

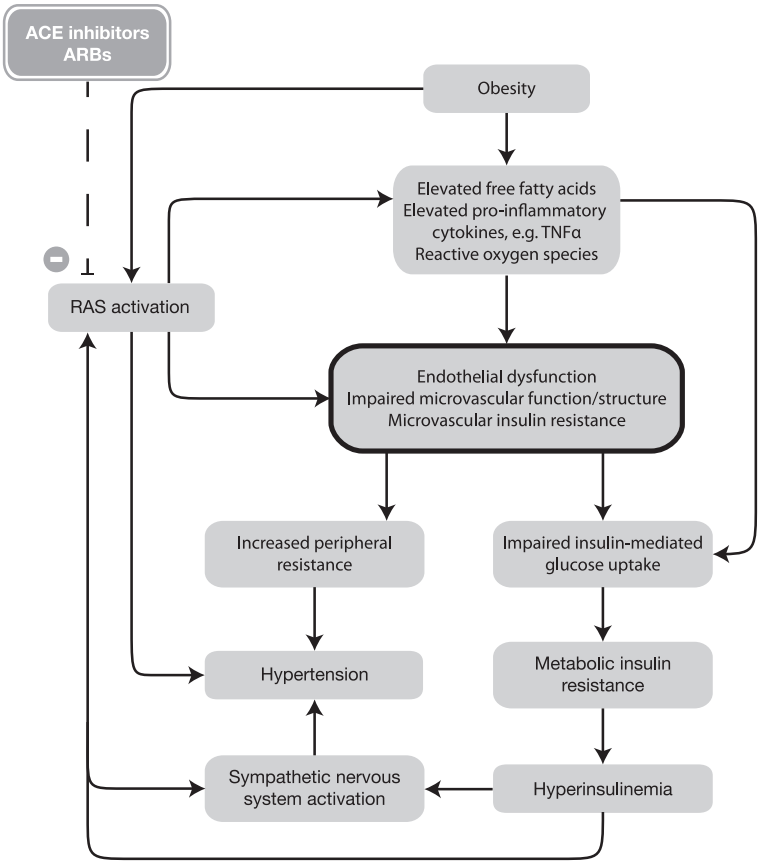

Figure 1.1. The postulated pathophysiological framework underlying the hypothesis that microvascular dysfunction links hypertension and insulin resistance

Pathophysiological mechanisms underlying microvascular insulin resistance

Factors that may be implicated in the pathogenesis of microvascular insulin resistance and dysfunction in insulin resistance and hypertension include overactivation of the RAS, chronic inflammation, and elevation in plasma free fatty acids (FFAs).

The renin-angiotensin system

The major biologically active end-product of the RAS is AngII. AngII is able to influence blood flow in the microvasculature via two G protein-coupled receptors, the type 1 receptors (AT_1R) and type 2 receptors (AT_2R).[96] Both AT_1Rs and AT_2Rs are present throughout the skeletal muscle microcirculation, including endothelial cells and vascular smooth muscle cells.[97] Activation of the AT_1R promotes vasoconstriction, inflammation, and proliferation whereas the AT_2R promotes vasodilation. AngII demonstrates both microvascular vasodilatory and vasoconstrictor effects depending on its relative actions on AT_1Rs and AT_2Rs, the examined tissue, the population studied, and the dosage and duration of administration.[98-101] In rat muscle, AngII induces net vasoconstriction, but increases oxygen uptake and glucose metabolism suggesting an increase in nutritive microvascular blood flow presumably by vasoconstriction of functional vascular shunts or predominately activating the AT_2Rs.[19,98,102,103] In humans, studies on the effects of AngII on microvascular blood flow distribution have not been performed, but most studies using total limb blood flow as an estimate of muscle perfusion have shown that acute AngII infusion increases muscle blood flow in healthy individuals despite a general vasoconstrictive effect measured as an increase in systemic pressure.[104-106] This action appears to reflect the fact that AngII increases vascular resistance more in renal and splanchnic vessels than in femoral vessels.[104,107] Notably, several studies demonstrated a dose-related increase in insulin-mediated glucose disposal in response to short-term (sub)pressor infusions of AngII in healthy individuals.[104-106,108-110] The capability of AngII to redirect blood away from tissues that respond minimally to insulin and toward tissues in which insulin stimulates glucose uptake (e.g. skeletal muscle), was suggested to be the underling mechanism for this increase in insulin sensitivity.[104-106,111]

However, the finding of increased insulin-mediated glucose disposal in healthy individuals with systemic AngII administration seems in contrast with the general finding that blockade of the RAS, by either angiotensin-converting enzyme (ACE) inhibitors or AngII AT_1R blockers (ARBs), enhances insulin-mediated glucose uptake in hypertensive and insulin-resistant individuals[112-116] and reduces the risk for new-onset diabetes in these patients.[117,118] As compared with AngII effects in healthy individuals, in insulin-resistant states, such as hypertension, obesity, and type 2 diabetes the stimulatory effect of AngII on insulin sensitivity and on skeletal muscle blood flow is impaired[106] and the pressor response to AngII is increased.[85,119] Thus, these data indicate that the actions of AngII might be opposite in insulin-resistant states compared to healthy individuals. This is most likely due to overactivity of the RAS with upregulation of AT_1R expression and/or activity as typically found in these conditions.[120-126] Activation of the RAS might impair insulin-mediated glucose uptake through the development of microvascular insulin resistance. Indeed, recent in vitro studies have suggested that AngII might negatively modulate insulin's actions via a

crosstalk at multiple levels of the insulin signaling cascade.[127,128] First, AngII interferes with insulin signaling by affecting insulin-induced tyrosine phosphorylation of IRS-1, impeding its interaction with PI3K.[127,129] Second, activation of AT_1Rs by AngII stimulates the production of ROS via NAD(P)H oxidase, which reduces NO bioavailability and is implicated in vascular inflammation.[84,130] Third, AngII can directly increase ET-1 release from endothelium thereby facilitating vasoconstriction.[131,132] Fourth, AT_1R activation induces inflammation via the release of proinflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) via the nuclear factor-kappaB (NFκB) pathway.[133,134] Finally, it has been proposed that AngII exhibits anti-adipogenic actions, thereby inhibiting adipocyte differentiation and elevating FFA levels[135] (Figure 1.2). Thus, in vitro studies demonstrate that there are multiple direct and indirect mechanisms for the RAS to contribute to vascular insulin resistance through the modulation of insulin signaling and other pathways in vascular tissue. There is also evidence from in vivo animal studies that the RAS is involved in the development of microvascular insulin resistance since RAS inhibition using the ACE inhibitor quinapril has been shown to restore the microvascular action of insulin in Zucker diabetic fatty rats.

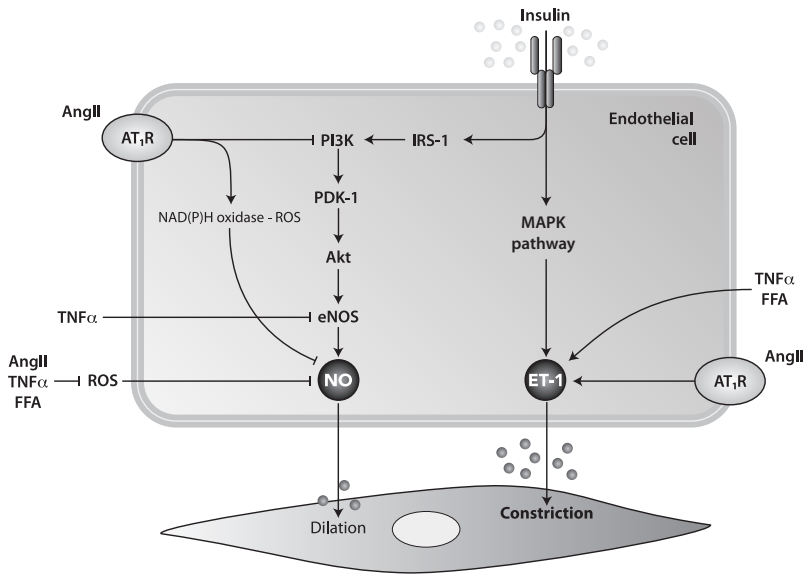

Figure 1.2. Insulin signaling pathways in the endothelium for the production of nitric oxide (NO) and endothelin-1 (ET-1). Angiotensin II (AngII), tumor necrosis factor-α (TNFα), and free fatty acids (FFA) inhibit the phosphatidylinositol 3-kinase (PI3K) pathway and stimulate the mitogen-activated protein kinase (MAPK) pathway via a crosstalk at multiple levels. IRS-1, insulin receptor subsrate-1; PDK-1, phosphoinositide-dependent kinase-1; Akt, protein kinase B; eNOS, endothelial nitric oxide synthase; AT_1R, angiotensin type 1 receptor ; ROS, reactive oxygen species.

Dyslipidemia and chronic inflammation

Evidence suggests that dyslipidemia and chronic inflammatory activation of adipose tissue might also be involved in the pathogenesis of microvascular insulin resistance. Adipose tissue, and in particular visceral adipose tissue, secretes a variety of bioactive substances including FFAs, hormones, or inflammatory cytokines.[136,137] FFAs and pro-inflammatory cytokines such as TNFα and IL-6 are abundantly present in the insulin-resistant state and vascular disease.[137] FFAs have been shown repeatedly to induce insulin resistance, inflammation and endothelial dysfunction.[138-142] In healthy humans and laboratory animals, acute elevation of plasma FFA concentration abrogated insulin-mediated muscle capillary recruitment using the insulin clamp and a mixed meal challenge, and this effect correlated well with inhibition of insulin-mediated glucose uptake.[30,37,38,143] Conversely, lowering FFA levels improved basal and insulin-mediated capillary recruitment in obese insulin-resistant individuals.[30] FFAs may induce insulin resistance in the vasculature through its ability to decrease IRS-1-associated PI3K activity and consequently insulin-mediated NO production,[144-145] or via increased release of the vasoconstrictor ET-1.[55,146,147] Moreover, it has been shown recently that FFAs can induce insulin-mediated vasoconstriction in muscle resistance arteries via activation of PKCθ and the subsequent inhibition of Akt and stimulation of ERK1/2.[148] In addition, FFAs may also contribute to endothelial dysfunction by inducing oxidative stress, by activating the NFκB pathway, or by triggering endothelial cell apoptosis and inhibiting cell cycle progression.[149-153]

Like FFAs, acutely administered TNFα directly impairs insulin-mediated capillary recruitment and insulin-mediated glucose uptake in rat muscle in vivo.[154,155] Actually, in isolated resistance arterioles, insulin has been shown to cause vasoconstriction in the presence of TNFα infusion.[156] In addition, it has been shown that TNFα associates with impaired capillary recruitment in human[157] and that weight loss resulted in significant amelioration of endothelial function that closely correlated with a reduction in circulating TNFα.[158] TNFα may induce endothelial insulin resistance via its ability to directly or indirectly induce insulin resistance in the PI3K pathway (e.g. via a p38 MAPK-dependent mechanism or JNK activation) and to stimulate the phosphorylation of ERK1/2.[156,159,160] In addition, TNFα may impair the balance between endothelial-derived vasodilator and vasoconstrictor substances by downregulation of the expression of eNOS,[64,161,162] upregulation of the expression of ET-1,[163] or ROS production.[164,165] Thus, circulating FFAs and TNFα are likely candidates to contribute to microvascular insulin resistance by modulating insulin signaling and transcription. In addition, the local secretion of adipokines from fat depots next to the vascular wall (i.e. perivascular adipose tissue) may have adverse effects on vasoregulatory properties.[166,167]

Concluding remarks

Microvascular insulin resistance is a potential factor explaining the clustering of hypertension, obesity, and insulin resistance, i.e. key features of the metabolic syndrome (Figure 1.1). Targeting mechanisms of microvascular dysfunction in prevention and therapy might therefore be important. Hence, clarification of pathophysiological pathways that contribute to microvascular dysfunction is essential. The RAS may be an important pathophysiological mechanism since the RAS itself regulates tissue perfusion, depending on its relative actions on AT_1Rs and AT_2Rs, and there is a crosstalk between AngII and insulin signaling pathways in the endothelium. However, to date the precise interaction between AngII and insulin in regulating microvascular perfusion in vivo in humans is still lacking. It is therefore necessary to investigate the effects of AngII administration and ACE inhibitors or ARBs, with concomitant insulin infusion on microvascular perfusion in both healthy individuals (i.e. individuals with a 'normal' regulated RAS) and in individuals with hypertension and/or insulin-resistance (i.e. individuals with an activated RAS).

Outline of the thesis

This thesis focuses on two objectives. First, this thesis describes two double-blind placebo-controlled randomized trials designed to investigate the role of the RAS in the regulation of insulin-induced microvascular perfusion and insulin-mediated glucose uptake in both healthy and hypertensive individuals. Second, this thesis examines the effects of meal ingestion and an oral glucose load on skin microvascular perfusion in both lean and obese (insulin-resistant) individuals to further explore the role of insulin-induced capillary recruitment in glucose metabolism in a physiological setting rather than during the insulin clamp. All investigations described in this thesis were performed in the skin microcirculation, using skin capillary dynamics as a model for muscle microcirculation. **Chapter 2** provides a detailed description of the measurement techniques used for the assessment of microvascular function and metabolic insulin sensitivity.

Part I

It has become clear that insulin-induced capillary recruitment plays a crucial role in insulin-mediated glucose uptake. The RAS is involved in the regulation of insulin-mediated glucose uptake. However, the effects of AngII on glucose metabolism are quite different in healthy individuals and insulin-resistant states; the underlying mechanisms are unknown. There is evidence that the RAS interacts with insulin to regulate tissue perfusion. However, in humans, the effects of AngII on insulin-induced capillary recruitment, as a mechanism to regulate insulin-mediated glucose uptake, have never been assessed. Therefore, in **chapter 3** the question is addressed whether the increase in insulin-mediated glucose uptake found with AngII infusion in healthy individuals is related to an AngII-induced stimulation of insulin-induced capillary perfusion. In addition, **chapter 4** examines the effects of acute ARB administration on insulin-induced capillary perfusion and insulin-stimulated glucose uptake in hypertensive, insulin-resistant individuals.

Part II

Although muscle is the main site of peripheral insulin-mediated glucose uptake, stimulatory effects of insulin on microvascular perfusion have been detected in both skin[24-29] and skeletal muscle.[30-32,34] Skin is the only site available in humans to examine capillary numbers directly, dynamically and noninvasively. Several studies in healthy individuals have shown that systemic hyperinsulinemia induced by the hyperinsulinemic euglycemic clamp technique increases the number of perfused skin capillaries[48,49] and expands the volume of microvasculature perfused in human forearm muscle.[24,28] In addition, it has been demonstrated that defects in the microvascular actions in skeletal muscle as well as skin are related to hypertension, obesity, and insulin resistance.[26,29,31,34,73] However, although the clamp technique provides an excellent assessment of responsiveness to

physiological hyperinsulinemia, it does not mimic the much more complex physiological responses that typically occur after the ingestion of a meal. Therefore, it is important to examine microvascular responses following meal ingestion used as a stimulus for endocrine insulin production. Recently, studies using contrast-enhanced ultrasound have demonstrated that mixed meal ingestion is a potent stimulus to increase perfused microvascular volume within skeletal muscle.[49-51] However, no data regarding the physiological microvascular response to meal feeding in skin are available. Therefore, **chapter 5** examines the effects of an oral glucose load and a mixed meal on microvascular vasomotion in both lean and obese individuals. Vasomotion is thought to play a role in ensuring optimal delivery of nutrient to the tissue[168] and it seems plausible that capillary recruitment may be mediated at least in part via effects on microvascular vasomotion. In **chapter 6** the effects of an oral glucose load and a mixed meal on skin capillary perfusion and endothelium-(in)dependent vasodilation in lean and obese individuals was assessed.

Finally, **chapter 7** provides an overview of the main conclusions based on the studies described in this thesis. These conclusions are put into a broader perspective and issues for future research are addressed.

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2

Methods

METHODS

This chapter provides information about all techniques and methods used in this thesis. Methods are subdivided in those used to examine microvascular function and those used to measure insulin sensitivity.

Microvascular function

The microcirculation is generally taken to include the arterioles, capillaries, and venules, each with their own characteristic structure and function.[1] Exchange of gases, nutrients, and metabolites between the blood and tissue occurs almost exclusively in the capillaries, whereas the precapillary arterioles are more involved in the regulation of local and overall peripheral vascular resistance.[1]

In the present thesis we focused on the microvascular effects of insulin and their role in glucose metabolism. Since muscle is the main peripheral site of insulin-mediated glucose uptake, assessment of muscle microvascular function would be most relevant. However, in the present thesis we studied the microvasculature in skin and not skeletal muscle, because the skin is an unique site allowing simple and reproducible assessment of capillary density and endothelial function. More precisely, the skin is the only site available in humans allowing direct non-invasive visualization of capillaries at rest and during provocative stimuli. In addition, the cutaneous microcirculation is considered a representative vascular bed to examine the mechanism of generalized systemic microvascular dysfunction.[2,3] This is supported by findings of cutaneous microvascular abnormalities in individuals with various cardiovascular risk factors and generalized systemic illnesses, such as diabetes and heart disease, and by the finding of parallel aging mechanisms in the cutaneous and the systemic vasculature.[2] Importantly, with regard to the specific effects of insulin, several studies have demonstrated comparable metabolic[4,5] and vascular[6-11] effects of insulin in muscle and skin. In addition, skin microvascular vasodilator capacity is associated with both insulin's vascular and metabolic actions in skeletal muscle.[10,12] Moreover, it has been demonstrated that the (systemic) effects of obesity and free fatty acids on insulin-mediated microvascular recruitment in muscle[7,8] can be reproduced in skin.[9,10] Thus, these data strongly suggest that the vascular responses observed in skin reflect those in muscle.

Anatomy and physiology of skin microcirculation

The skin microcirculation consists of two vascular beds; the nutritive capillaries and arteriovenous anastomoses.[13] The deeper arteriovenous anastomoses are communications between small arteries or arterioles and the corresponding venous channels and serve a thermoregulatory function. They are especially numerous in fingertips, ears and nose, whereas the skin of the dorsal finger and forearm are considered devoid of arteriovenous anastomoses.[14] The nutritional capillaries are the most superficial vessels located at a close

distance from the skin surface. In the nailfold area the capillary loops run parallel to the skin surface, whereas in other (i.e. more proximal) areas the nutritional capillary loops run perpendicular to the skin surface and only the apex of the capillary loops can be visualized. It is the nutritive capillaries that are responsible for the maintenance of tissue viability; they are the source of oxygen, nutrient, and fluid exchange and capillary density determines the diffusion distance for these materials within the tissues.[13] As described in **chapter 1** it is assumed that not all skin capillaries are perfused at any one time; i.e. some are perfused while others are shut down depending on whether their parent arteriole is "open" or not.[15-17] Previous studies have demonstrated that the time that capillaries are filled with erythrocytes varies greatly among different capillaries in the same area, as some capillaries seem continuously filled with erythrocytes while others are intermittently perfused.[17]

Methods used to study the microvasculature

In this thesis we used different methods to study the skin microvasculature; (1) capillary microscopy, (2) laser Doppler flowmetry in combination with iontophoresis of acetylcholine and sodium nitroprusside or Fourier analysis of the laser Doppler signal. All microvascular measurements were conducted after 30 min of acclimatization in a quiet, temperature-controlled room (T=23.4 ± 0.5°C) with the subjects in supine position and the investigated hand at heart level. All measurements were performed after a 10h fast and all subjects abstained from drinking alcohol for a period of 24h before each study day and from performing strenuous exercise for a period of 48h before each study day. Skin temperature was monitored continuously during all tests.

1. Capillary microscopy

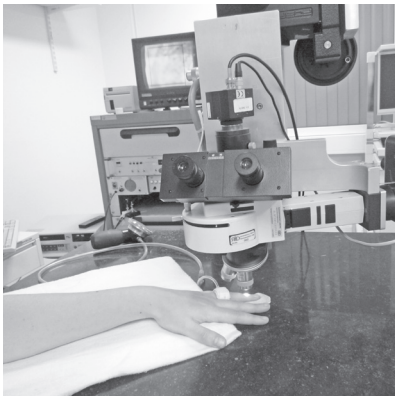
Intravital capillary microscopy is a dynamic method to directly visualize perfused nutritive capillaries in human skin. Capillary microscopy without dyes depends on the presence of red blood cells inside capillaries for their identification. In our studies nailfold capillaries in the dorsal skin of the fourth finger were visualized by a capillary microscope (Leitz-Orthoplan) in combination with a CCD-camera. To visualize the capillaries, a 4.0 objective (Leitz N.A. 0.14) was used with a total system magnification of 160x (Figure 2.1A). The investigated finger was immobilized in a mass of clay and paraffin oil was applied to the area of observation to reduce skin reflections and improve skin translucency. Capillaries were visualized ~4.5 mm proximal to the terminal row of capillaries in the middle of the nailfold. This distance was the width of three visual fields and here capillaries run perpendicular to the skin. Subsequently, a characteristic capillary (i.e. a capillary that was constantly perfused and had an eye-catching morphological feature) was kept on the same spot of the visual field (marked by a dot on the monitor) to ensure that capillary density was measured in the exact same visual field during the entire experiment. Three variables

were measured: (1.1) baseline capillary density, (1.2) hyperemic (functional) capillary recruitment, and (1.3) capillary density during venous congestion. All procedures were performed on two separate visual fields of 1 mm^2 and the mean of both measurements was used for analyses. Capillaries were counted offline from a freeze-framed reproduction of the videotape and from the running videotape when it was uncertain whether a capillary was present or not. Capillaries were counted by one or two investigator(s) who were blinded to the experimental status of the recordings.

1.1 Baseline capillary density

Baseline capillary density was defined as the number of erythrocyte-perfused capillaries per square millimeter of nailfold skin. The number of capillaries at baseline was counted during a 30-second period. During this period, only continuously perfused capillaries were counted, although intermittently perfused capillaries were also visible (Figure 2.1B).

A



B

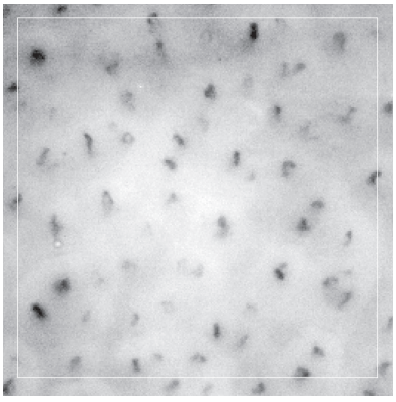


Figure 2.1. A: capillary microscopy equipment, with cuff applied on the base of the fourth finger. B: Microscopic image obtained - baseline capillary density.

1.2 Hyperemic (functional) capillary recruitment

Since intravital microscopy does not show nonperfused capillaries as it relies on the presence of erythrocytes, post-occlusive reactive hyperemia after 4 min of arterial occlusion was used to assess the functional capillary reserve capacity.[16,17] For this, a miniature cuff (Digit cuff, Hokanson, Bellevue, WA, USA) was applied on the base of the fourth finger and inflated to suprasystolic pressure (260 mmHg) (Figure 2.1A). The same characteristic capillary was kept on the same spot as during recordings of baseline capillary density to ensure that that capillary density in the resting state and during post-occlusive reactive hyperemia were measured in the same area. After 4 min of arterial occlusion the cuff was released. During this procedure the image was constantly kept in focus and the response

was stored on videotape. The number of capillaries was counted directly after the release of the cuff for a period of 45 s (the major part of the increase in capillary number occurs instantaneously).

1.3 Venous congestion

To expose a maximal number of capillaries we applied venous congestion, with the digital cuff inflated to 60 mmHg for 60 s (as an estimate of the total (structural) capillary reserve). Capillaries during venous congestion were counted in the 60-second recordings. Venous congestion allows the trapping of red cells in plasma-only perfused capillaries as well as intermittently perfused capillaries.[18] The venous backpressure reduces the pressure gradient driving flow, which by temporarily reducing flow and washout of vasodilators causes vasodilatation.[18] Prolonged venous occlusion on the other hand, could increase precapillary resistance through the venoarteriolar reflex.[17,19] However, we avoided prolonged venous occlusion (i.e. 60 s) and we examined the capillaries with the hand held at heart level, which abolishes the effects of this reflex. Nevertheless, it should be kept in mind that only erythrocyte-filled capillaries are visualized with capillary microscopy as a result of which the true maximal number of capillaries might be underestimated.

2. Laser Doppler flowmetry

Laser Doppler flowmetry in combination with iontophoresis of acetylcholine and sodium nitroprusside was used to asses endothelium-dependent and endothelium-independent vasodilation of the skin microcirculation (2.1). Fast Fourier analysis of the laser Doppler flow signal was used to perform vasomotion analysis (2.2).

Laser Doppler flowmetry is a non-invasive technique which allows continuous monitoring of the dynamic variation in microvascular perfusion in the skin. The technique is based on the emission of a beam of laser light carried by a fiber-optic probe. The light penetrates the skin and a fraction of the light is backscattered by moving erythrocytes and also partly absorbed by the tissue being studied. Light which is backscattered from moving erythrocytes undergoes a shift in frequency proportional to their velocity, according to the Doppler principle, while light hitting static objects is unchanged (Figure 2.2). The magnitude and frequency distribution of these changes in wavelength are directly related to the number and velocity of the blood cells in the sample volume. The information is picked up by a returning fiber, converted into an electronic signal and analyzed. A limitation of Laser Doppler flowmetry is that researchers cannot be sure what volume of tissue is being measured. Thus, rather than perfusion being measured quantitatively (e.g. ml/min/100 gram tissue), it is expressed as blood flow in arbitrary Perfusion Units (PU).[20] PU is the product of the velocity and the concentration of moving red blood cells.[21]

The measuring depth of the laser Doppler technique depends on tissue properties such as the structure and density of the capillary beds, pigmentation, oxygenation, etc. It also

depends on the wavelength of the laser light and on the distance between the sending and receiving fibers in the laser Doppler probe. In our studies, a probe with standard fiber separation and a 780 nm wavelength laser was used (Periflux 5000, Perimed, Stockholm, Sweden) with a measuring dept in the order of 0.5-1.0 millimeter. The laser Doppler signal will therefore be derived from arterioles, venules, and nutritive capillaries (derived from http://www.perimed-instruments.com/support/theory/laser-doppler).

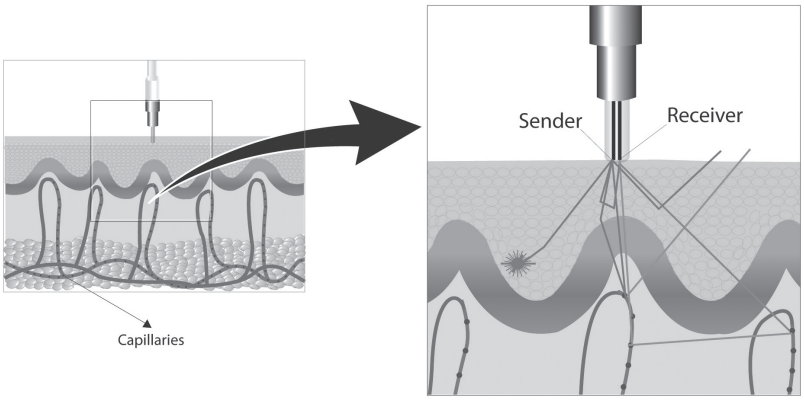


Figure 2.2. Principle of laser Doppler flowmetry. A laser beam penetrates the light and is backscattered by moving blood cells. This generates a signal proportional to tissue flow.

2.1 Endothelium-dependent and endothelium-independent vasodilation

Iontophoresis is defined as the introduction of ions of soluble salts into tissues by means of a direct electrical current. The basic principle of iontophoresis is that molecules of drugs in solution that are positively or negatively charged will migrate across the skin under the influence of an applied current according to the rule that like charges repel each other. Thus, positively charged drug ions can be non-invasively introduced through the skin under a positively charged electrode (anodal iontophoresis), whereas negatively charged drug ions can be introduced under a negatively charged electrode.[20] The amount of drug delivered is dependent on the magnitude and the duration of the current applied; the quantity of drug delivered is negligible to have any systemic side-effects.

In this thesis we used the Laser Doppler technique in combination with iontophoresis of the vasodilators acetylcholine and sodium nitroprusside for the examination of skin microvascular endothelial and smooth muscle cell function. Acetylcholine is the standard test drug for the assessment of endothelial function, although other drugs, such as methacholine, bradykinin, and substance P, can also be introduced via iontophoresis.[22] The mechanism by which acetylcholine induces endothelium-dependent vasodilation is

cause for some debate, but may be mediated by the endothelium-dependent production of nitric oxide (NO),[23-25] prostaglandins,[26,27] and/or endothelium-derived hyperpolarizing factor.[20] Sodium nitroprusside is an endothelium-independent vasodilator. It is a NO donor, acting directly on smooth muscle cells to induce relaxation through an increase in cyclic guanosine monophosphate.[20,22,28] A reduction in the vascular response to acetylcholine with no concurrent reduction in sodium nitroprusside response is indicative of endothelial dysfunction. A reduction in response to sodium nitroprusside can be interpreted as a structural change within the vessel causing a reduction in the vasodilator capacity of the vessel.[20]

Investigational protocol - skin endothelium-(in)dependent vasodilation

To start, a disposable gel sponge in the drug delivery electrode (thermostatic laser Doppler probe) was soaked with drug solution (~200 µl). Subsequently, the skin was cleaned with isopropyl alcohol and the drug delivery electrode was fixed on the middle phalanx of the second finger (acetylcholine) or the middle phalanx of the fourth finger (sodium nitroprusside) of the left hand. The dispersive (i.e. reference) electrode was fixed at the wrist, ~15 cm away from the drug delivery electrode to complete the circuit and polarity adapted to the electric charge of the vasoactive molecule. Both leads of the iontophoresis device were attached to the electrodes.

After recording 1 min of stable baseline blood flow, acetylcholine (1% Miochol, Novartis Pharma GmbH, Nürnberg, Germany) was delivered using an anodal current; seven doses of 0.1 milliamps (mA) for 20 s with a 60-s interval. Sodium nitroprusside (0.01% Nipride, Radboud Hospital, Nijmegen, The Netherlands) was delivered using a cathodal current; nine doses of 0.2 mA for 20 s with a 90-s interval. Iontophoresis was performed with a thermostatic laser Doppler probe heated to 30°C. The increase in perfusion (PU) due to acetylcholine or sodium nitroprusside (from baseline to maximal perfusion) was used for further analysis.

2.2 Microvascular vasomotion

Vasomotion is known as oscillations in small vessel lumens resulting from smooth muscle dilatation and constriction, a phenomenon that has been observed in many microvascular beds. It is thought that modulation of vasomotion by vasoactive substances released by metabolizing tissue plays a role in ensuring optimal delivery of nutrient to the tissue.[29] Moreover, there is evidence that insulin is involved in the regulation of microvascular vasomotion.[30-32] Different mechanisms might contribute to vasomotion; the vascular endothelium, the neurogenic activity of the vessel wall, the intrinsic myogenic activity of vascular smooth muscle, respiration, and heart beat. In the present thesis we were interested in the effects of a glucose drink and a mixed meal drink on skin microvascular

vasomotion. In order to perform vasomotion analyses, skin blood flow was measured with a thermostatic laser Doppler probe (PF 457, Perimed, Stockholm, Sweden) at the dorsal side of the wrist of the right arm using the Periflux 5000 laser Doppler system (Perimed, Stockholm, Sweden). Signals were recorded for 30 min, both at baseline and 30 min after intake of the drinks, with a sample frequency of 32Hz. Fast Fourier transform analysis was performed by means of Perisoft dedicated software (PSW version 2.50, Perimed, Sweden) in order to determine the contribution of the five frequency components to the variability of the laser Doppler signal (i.e. endothelial, 0.01-0.02 Hz; neurogenic, 0.02-0.06 Hz; myogenic, 0.06-0.15 Hz; respiratory, 0.15-0.40 Hz; and heart beat, 0.40-1.60 Hz).

Methods used to study insulin sensitivity

In this thesis all plasma insulin concentrations were measured by radioimmunoassay technique (AutoDELFIA, PerkinElmer, Massachusetts, USA). Blood glucose concentrations were determined with a glucose analyzer YSI2300 (Yellow Springs Instrument, Yellow Springs, OH, USA).

1. Hyperinsulinemic euglycemic clamp technique [partly adapted from 33]

There is general agreement that the hyperinsulinemic euglycemic clamp is the best available method for the measurement of insulin-mediated peripheral glucose uptake.[33] The hyperinsulinemic clamp methodology has been developed and widely studied by DeFronzo et al.[34] With the insulin clamp technique, exogenous insulin is administered as a prime followed by a constant intravenous infusion at a rate designed to maintain a pre-set hyperinsulinemic plateau; simultaneously, the plasma glucose concentration is clamped at the normal fasting (~5 mmol/l, euglycemic) or any pre-existing (isoglycemic) level by means of administering a variable infusion of glucose intravenously.[33] Generally, steady-state insulin concentrations are reached within 30-50 min after initiation of insulin infusion[33,35] and hepatic glucose release and endogenous insulin production are completely suppressed at the insulin concentrations reached during this steady-state.[33,35] As a result, when steady-state is attained, the exogenous glucose infusion rate equals the amount of glucose disposed by all the tissues in the body and thus provides a quantitation of overall insulin sensitivity.[33] The less glucose that needs to be infused to sustain euglycemic glucose levels the greater the level of insulin resistance. The main limitations of the insulin clamp are that it is time consuming, invasive (two intravenous lines), labour intensive and expensive, and that it requires an experienced operator to manage the technical difficulties.[36]

In this thesis we applied the hyperinsulinemic euglycemic clamp technique to induce steady-state hyperinsulinemia to examine effects of angiotensin II infusion and angiotensin II AT_1-receptor blockade on insulin-induced capillary recruitment and insulin-mediated glucose uptake **(chapter 3 and 4)**. In our studies insulin (Actrapid, Novo Nordisk, Bagsvaerd,

Denmark) was infused at a rate of 50 mU/kg/h. Mean fasting glucose concentrations were determined from three glucose concentrations measured before the start of the clamp. Isoglycemia was maintained by adjusting the rate of a 20% D-glucose infusion based on plasma glucose measurements performed at 5-10 min intervals. Whole-body glucose uptake (M) was calculated from the glucose infusion rates. M was expressed per kg body weight per unit of plasma insulin concentration (M/I), thereby correcting for differences in steady-state insulin concentrations.[33] For convenience, the M/I ratio was multiplied by 100.

2. Oral glucose tolerance test

The oral glucose tolerance test (OGTT) is a simple test to diagnose glucose intolerance and type 2 diabetes.[37] After an overnight fast, blood samples for determinations of glucose and insulin concentrations are taken at preset time points following a standard oral glucose load (75 g).[37] In **chapter 5 and 6** we examined skin microvascular responses to an OGTT and compared these responses to the microvascular responses after mixed meal ingestion. We also compared the plasma glucose and insulin levels after the OGTT and mixed meal. In addition, we used the OGTT to identify and exclude diabetic individuals (i.e. 2h glucose level >7.8 mmol/l). In our studies blood samples were taken before ingestion of the glucose load (duplicate sample) and at 10, 20, 30, 40, 50, 60, 75, 90, 120, 150, and 180 min after ingestion of the glucose load.

3. Homeostatic model assessment

Homeostatic model assessment (HOMA) is a simple surrogate index for assessing insulin sensitivity/resistance. The HOMA model compares favorably with other models and has the advantage of requiring only fasting plasma samples assayed for insulin and glucose. There is good correlation between estimates of insulin resistance derived from HOMA and from the hyperinsulinemic euglycemic clamp.[38] HOMA1, the original model, contains a simple mathematical approximation of insulin sensitivity in which the product of the fasting values of insulin (FPI) and glucose (FPG) are divided by a constant: (FPIxFPG)/22.5.[39] Recently, the HOMA1 model was updated with some physiological adjustments to a computer model (HOMA2) with nonlinear solutions that provide a more accurate index of insulin sensitivity. In **chapter 5 and 6** of this thesis we used HOMA2 to asses insulin sensitivity in lean and obese individuals. For HOMA2 no cut-off point for detecting insulin resistance is available, however the HOMA2-value increases as the degree of insulin resistance increases which allows for comparison between lean and obese individuals.

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3

Angiotensin II enhances insulin-stimulated whole-body glucose disposal but impairs insulin-induced capillary recruitment in healthy volunteers

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Abstract

Background and aims - Angiotensin II increases insulin-mediated glucose uptake in healthy individuals. The underlying mechanisms are undefined. Angiotensin II may increase glucose uptake through a direct effect on muscle cell insulin signaling or through increasing insulin delivery to muscle cells through effects on the microvasculature. Our objective was to determine whether angiotensin II increases insulin-mediated glucose uptake through effects on insulin-induced capillary recruitment.

Methods - We examined the effects of angiotensin II on hyperinsulinemia-induced capillary density by measuring skin capillary density, capillary recruitment, and capillary density during venous congestion in 18 healthy subjects in the basal state, during systemic hyperinsulinemia, and during hyperinsulinemia with coinfusion of angiotensin II or phenylephrine (pressor control). In addition, whole-body glucose uptake and blood pressure were measured.

Results - Capillaroscopy data of 13 subjects were available for analysis. Compared with the basal state, hyperinsulinemia increased baseline capillary density (51.5±9.0 vs. 55.2±10.8 n/mm^2, P<0.01), capillary recruitment (67.8±6.8 vs. 70.6±7.5 n/mm^2, P < 0.05), and capillary density during venous congestion (78.5±12.0 vs. 80.3±12.0 n/mm^2, P<0.01). Infusion of angiotensin II, but not phenylephrine, reduced insulin-induced capillary recruitment (69.3±8.6 vs. 65.2±8.0 n/mm^2, P<0.05) and capillary density during venous congestion (79.7±15.3 vs. 73.9±12.1, P<0.05) while enhancing glucose uptake [2.40±0.7 vs. 2.68±0.6 (mg/kg/min per pmol/l) × 100, P<0.01)] (n=18).

Conclusion - Angiotensin II increases insulin-mediated glucose uptake in healthy individuals. This increase was probably not related to increases in microvascular perfusion because infusion of angiotensin II during hyperinsulinemia reduced insulin-mediated skin capillary recruitment. Additional studies are needed to investigate whether angiotensin II directly affects insulin delivery through increasing insulin transport across the microvasculature.

Introduction

Angiotensin II (AngII) is involved in the regulation of insulin-mediated glucose uptake.[1,2] However, the effects of AngII appear to be complex and to differ between healthy individuals on the one hand and individuals with hypertension, obesity, or type 2 diabetes on the other.[3–6] For example, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers, both of which decrease the effects of AngII, have been shown to increase insulin-mediated glucose uptake in individuals with hypertension, obesity, or type 2 diabetes,[4,6-9] whereas, paradoxically, acutely raising AngII systemically in healthy individuals has been shown to increase insulin-mediated glucose uptake.[3,10–12] On the other hand, studies with ACE inhibition in healthy non-obese subjects have demonstrated no increase[13,14] or even a decrease[15] in insulin-mediated glucose uptake. How AngII affects glucose metabolism remains to be defined.

Two effects of insulin are crucial for stimulating glucose uptake in muscle. First, through its membrane receptor, insulin has direct effects on muscle cells. Second, insulin regulates its own delivery, and that of glucose, to muscle cells through actions on the microvasculature.[16,17] Muscle microvasculature consists of nutritive capillary networks, which perfuse working muscle cells, and of nonnutritive networks, which are thought to be involved in thermoregulation.[18,19] By relaxation of terminal arterioles connected to nutritive capillary networks, insulin can alter the distribution of muscle blood flow, resulting in enhanced access of glucose and insulin to muscle cells.[16,17] Indeed, increasing or decreasing nutritive flow has been shown to result in parallel changes in insulin-mediated glucose uptake.[20–23]

Because microvascular perfusion appears to be crucial in the regulation of muscle glucose metabolism and AngII receptors are present in muscle microvasculature, the question thus arises whether vasoconstrictors such as AngII can affect insulin-mediated glucose uptake through influencing insulin-induced nutritive capillary recruitment. Possible effects of AngII on microvascular perfusion have been examined in animal studies. In a rat model in which the hind limb was perfused with perfusate, AngII infusion increased the number of perfused capillaries in muscle.[19] In an *in vivo* rat study, AngII infusion increased muscle microvascular blood volume.[24] However, in these studies, the effects of AngII were determined either in the absence of insulin[19] or at fasting plasma insulin levels,[24] whereas most glucose uptake takes place postprandially, i.e. during hyperinsulinemia. To date, there have been no studies (i.e. in humans or animals) where the effects of AngII on capillary recruitment during hyperinsulinemia have been determined.

The present study was designed to determine, in healthy subjects, whether AngII infusion enhances insulin-induced capillary density and therefore insulin-mediated glucose uptake in healthy individuals, using skin capillary dynamics as a model for muscle microcirculation.[22,25,26]

Subjects and Methods

Subjects

Eighteen healthy, non-obese (body mass index <27 kg/m^2) Caucasian subjects participated in this study (Table 3.1). Participants were healthy as judged by medical history, nondiabetic,[27] normotensive (<135/<85 mmHg) as determined by ambulatory 24h blood pressure monitoring (SpaceLabs 90207, Redmond, WA), and non-smokers. Participants did not use any medication except for one subject using oral contraceptives. All participants gave informed consent for participation in the study. The study was undertaken with the approval of the local ethics committee and performed in accordance with the Declaration of Helsinki.

Study design

All subjects were allocated to two interventions in random order, i.e. a euglycemic hyperinsulinemic clamp with either 2 ng/kg/min AngII infusion or with 0.5 µg/kg/min phenylephrine (PE) infusion (Figure 3.1). Investigators were blinded with regard to the pressor infused (in a control experiment, at basal insulin levels, pressor doses were tested to be equivalent, n=7). To confirm that short-term nonspecific changes in microcirculatory function are small,[28] in addition, a time- and volume-control experiment was performed in a fashion identical to the clamp experiment with the infusion of the same amounts of fluid (0.65% saline) and microvascular measurements at the same time intervals, but without insulin or glucose infusion or AngII or PE infusion. The interval between each study day was 7 days. To standardize and suppress the activity of the endogenous renin-angiotensin system, subjects adhered to a high-sodium diet (220 mmol/day) for 1 wk before the first visit and also before the following visits. Compliance with the diet was assessed by measurements of 24h urinary sodium excretion.

All measurements were conducted in a temperature-controlled room (23.4±0.5 °C) at 08.00 a.m., after a 10h fast, with the subjects in the supine position. Subjects were asked to refrain from drinking alcohol for a period of 24h before each study day and to perform no strenuous exercise for a period of 48h before each study day. Baseline measurements were obtained after allowing 30 min of rest and acclimatization after the insertion of two polytetrafluoroethylene catheters (Venflon; Viggo, Gotenborg, Sweden). Skin temperature was monitored during the tests.

In addition to the three experimental days described in Figure 3.1, a subgroup of eight subjects underwent a 5.5h euglycemic hyperinsulinemic clamp without coinfusion of AngII or PE (hyperinsulinemic time-control).

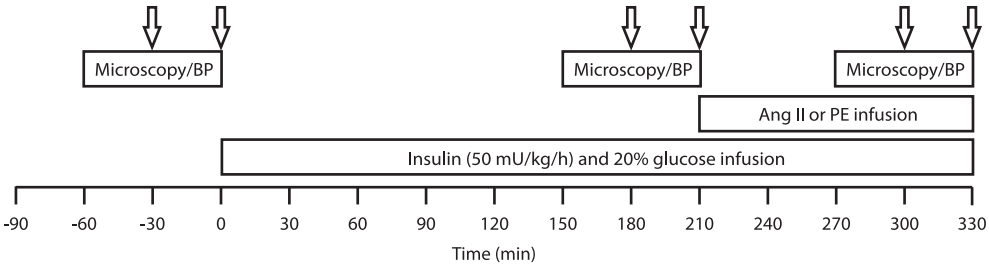

Figure 3.1. Design of the study. There were two interventions: hyperinsulinemic clamp with AngII infusion and hyperinsulinemic clamp with PE infusion. Microscopy indicates capillary microscopy of the skin of the finger; BP, blood pressure measurements. Arrows indicate blood samples for measurements of insulin concentrations. In addition, a time- and volume-control experiment was performed (infusion of 0.65% saline), with capillary microscopy measurements, blood pressure measurements and blood samples at the same time intervals as the hyperinsulinemic clamp days.

Hyperinsulinemic euglycemic clamp

Insulin sensitivity was assessed by the hyperinsulinemic euglycemic clamp method, using a modification of the method described by DeFronzo et al.[29] Briefly, insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused in a primed continuous manner at a rate of 50 mU/kg/h. Mean fasting glucose concentrations were determined from glucose concentrations measured at t = –60, –30, and 0 min. Normoglycemia was maintained by adjusting the rate of a 20% D-glucose infusion based on plasma glucose measurements performed at 5-10 min intervals. Whole-body glucose uptake (M value) was calculated from the glucose infusion rates during the last 60 min of hyperinsulinemia (t = 150–210 min) and the last 60 min of hyperinsulinemia plus AngII and PE infusion (t = 270–330 min). Similar time periods were used for the calculation of M during the 5.5h hyperinsulinemic time control without coinfusion of AngII or PE. M was expressed per kilogram body weight per unit of plasma insulin concentration (M/I), thereby correcting for differences in steady-state insulin concentrations. For convenience, the M/I ratio was multiplied by 100. Mean insulin concentrations during the hyperinsulinemic clamp were determined from steady-state insulin levels measured at t = 180 and 210 min. Mean insulin concentrations during AngII and PE infusion and during the hyperinsulinemic time-control experiment were determined from steady-state insulin levels measured at t = 270 and 330 min.

Capillary microscopy

Nailfold capillary studies were performed at baseline (t = 60 min) and during hyperinsulinemia (t = 150 min) with AngII or PE infusion (t = 270 min), as previously described.[30] Baseline capillary density was defined as the number of continuously erythrocyte-perfused capillaries per square millimeter of nailfold skin. Postocclusive reactive hyperemia after 4 min of arterial occlusion was used to assess the functional

reserve capacity (capillary recruitment).

In addition, we applied venous congestion to expose a maximal number of capillaries.[31] All procedures were performed twice, and the mean of both measurements was used for analyses. The day-to-day coefficients of variation of baseline capillary density, functional capillary recruitment, and number of capillaries after venous congestion were 6.5, 7.2, and 7.8%, as determined in 12 subjects on two separate days.

Blood pressure

During study days, blood pressure and heart rate (HR) were determined as depicted in Figure 3.1 (Datascope Accutorr Plus, Paramus, NJ).

Biochemical measurements

Plasma insulin concentrations were measured by RIA techniques (AutoDELFIA; PerkinElmer, Waltham, MA). Blood glucose concentrations were determined with a glucose analyzer YSI2300 (Yellow Springs Instrument, Yellow Springs, OH).

Statistical analysis

For the primary endpoint of our study (capillary recruitment), we calculated a sample size of n=13 to be sufficient to find a 10% change in capillary recruitment with a two-sided α-level of 0.05 and a power of 90%. In addition, it was calculated that this number would suffice to show the (expected) increase in insulin-mediated glucose uptake with AngII. Due to technical difficulties with the video microscopy, we needed to perform experiments in 18 subjects to obtain technically readable microscopic images in 13 subjects.

Data are expressed as mean ± SD. To study effects of hyperinsulinemia on microvascular function and blood pressure, mean values during the basal state (t = 0 min) and hyperinsulinemia (t = 210 min) of two clamp days were used (Figure 3.1). The Wilcoxon signed rank test for paired data was used to study effects of hyperinsulinemia, AngII, and PE on skin microvascular function. To minimize the effects of day-to-day variation, the effects of AngII and PE were tested within a clamp day.

A paired Student's t test was used to study effects, vs. the control study, of hyperinsulinemia, AngII, and PE infusion on blood pressure and to study the effects of AngII and PE infusion on insulin sensitivity (M/I). A two-tailed P value <0.05 was considered significant. All analyses were performed using the statistical software package SPSS version 15.0.

Results

Subjects

Baseline characteristics of the five individuals in whom no microvascular images were obtained were not different from the group as a whole (i.e. n=18) (data not shown). Characteristics of the total study population are shown in Table 3.1. The participants were healthy, middle-aged, normotensive subjects with normal fasting glucose and insulin levels. The urinary sodium excretion data showed compliance with the high-sodium diet.

Table 3.1. Characteristics of the study population

Number (men/women)	18 (8/10)
Age (yr)	49.4 ± 8.5
Body mass index (kg/m^2)	24.5 ± 2.1
Waist-to-hip ratio (men)	0.89 ± 0.06
Waist-to-hip ratio (women)	0.76 ± 0.05
SBP daytime, 24h-ABPM (mmHg)	121 ± 8
DBP daytime, 24h-ABPM (mmHg)	78 ± 5
Fasting plasma glucose (mmol/l)	5.0 ± 0.4
Fasting insulin (pmol/l)	27.4 ± 9.1
Insulin sensitivity (M/I value)	2.40 ± 0.71
Urinary sodium excretion (mmol/24h)	233.6 ± 84.4

Data are means ± SD. M/I value is expressed in (mg/kg/min per pmol/l) × 100. ABPM, Ambulatory blood pressure monitoring.

Effects of insulin on capillary density

Baseline capillary density, capillary recruitment, and density during venous congestion were comparable before (t = 0 min) the insulin or saline (time/volume control) infusion (52.8±11.2 vs. 54.2±9.6, 68.7±8.7 vs. 70.1±12.2, and 80.0±12.7 vs. 83.5±15.9 per mm^2, respectively). The effects of insulin on capillary densities are shown in Table 3.2. Hyperinsulinemia significantly increased baseline capillary density, (hyperemic) capillary recruitment, and density during venous congestion (Table 3.2). During the time- and volume-control day, no time- and volume-dependent effects on microvascular variables were found (data not shown).

Table 3.2. Nailfold capillary densities before and during the hyperinsulinemic clamp (mean values of two clamp days) (N=13)

	Basal insulin levels t = 0	Hyperinsulinemia t = 210
Baseline density (n/mm^2)	51.5 ± 9.0	55.2 ± 10.8 *
Capillary recruitment PRH (n/mm^2)	67.8 ± 6.8	70.6 ± 7.5 †
Venous congestion (n/mm^2)	78.5 ± 12.0	80.3 ± 12.0 *

Data are mean ± SD. n/mm^2, number of perfused capillaries per mm^2. PRH, peak reactive hyperemia. * P<0.01, † P<0.05 for basal insulin levels vs. hyperinsulinemia.

Effects of AngII and PE on capillary density during hyperinsulinemia

Hyperinsulinemia-associated baseline capillary density decreased during infusion of AngII and increased during infusion of PE (Table 3.3; P<0.03 for the change during AngII vs. the change during PE). In addition, infusion of AngII, but not PE, significantly reduced hyperinsulinemia-associated capillary recruitment and density during venous congestion (Table 3.3; P<0.01 for the change during AngII vs. the change during PE). Skin temperature was not significantly different during the experiments (30.9±1.0 ºC).

Table 3.3. Nailfold capillary densities during the hyperinsulinemic clamp and subsequent AngII or PE infusion (N=13)

	AngII day		PE day	
	Hyperinsulinemia *t* = 210	Hyperinsulinemia + AngII *t* = 330	Hyperinsulinemia *t* = 210	Hyperinsulinemia + PE *t* = 330
Baseline density (n/mm^2)	54.0 ± 11.5	51.8 ± 10.5	56.4 ± 13.4	60.2 ± 11.9 ‡
capillary recruitment PRH (n/mm^2)	69.3 ± 8.6	65.2 ± 8.0 *	71.8 ± 11.4	73.2 ± 12.3 †
Venous congestion (n/mm^2)	79.7 ± 15.3	73.9 ± 12.1 *	80.9 ± 13.4	82.2 ± 14.1 †

Data are mean ± SD. n/mm^2, number of perfused capillaries per mm^2. PRH, peak reactive hyperemia. * P<0.05 for hyperinsulinemia + AngII vs. hyperinsulinemia on the same day. † P<0.01, ‡ P<0.03 for increase during PE (*t* = 330 vs. *t* = 210 on PE day) vs. decrease during AngII (*t* = 330 vs. *t* = 210 on AngII day).

Effects of AngII and PE on blood pressure and HR during hyperinsulinemia

Hemodynamic variables during the clamp are shown in Table 3.4. Hyperinsulinemia induced a nonsignificant decrease in mean arterial pressure (MAP) and diastolic blood pressure (DBP). During hyperinsulinemia, both AngII and PE infusion markedly increased MAP, systolic blood pressure (SBP), and DBP. AngII infusion also significantly increased HR. The effects of AngII on MAP, SBP, DBP, and HR during hyperinsulinemia were significantly larger than those of PE during hyperinsulinemia (Table 3.4). In contrast, in the control experiment at basal insulin levels (n=7), the effects of AngII and PE on MAP, SBP, DBP, and HR were equivalent (MAP AngII +13.8±7.0 vs. PE +13.4±4.6 mmHg, P=0.76; SBP AngII +16.4±10.8 vs. PE +17.6±3.9 mmHg, P=0.76; DBP AngII +10.4±7.9 vs. PE +10.7±4.0 mmHg, P=0.56; HR AngII –1.3±4.6 vs. PE –3.4±6.4 bpm, P=0.32). The larger pressor response to AngII compared with PE at hyperinsulinemia was due to significant lower increases in MAP, SBP, and DBP with PE during hyperinsulinemia than during basal insulin levels (MAP +5.3±4.7 vs. +13.4±4.6 mmHg, P=0.001; SBP +9.5±7.7 vs. +17.6±3.9 mmHg, P=0.01; DBP +3.6±5.2 vs. +10.7±4.0 mmHg, P=0.01), compared with similar responses to AngII infusion during hyperinsulinemia and basal insulin levels. Blood pressure and HR did not change during the time- and volume-control day (data not shown).

Table 3.4. Hemodynamic changes during the hyperinsulinemic clamp (mean values of two clamp days), AngII and PE infusions (N=18)

	Basal insulin levels *t* = 0	Hyperinsulinemia *t* = 210	Hyperinsulinemia + AngII *t* = 330	Hyperinsulinemia + PE *t* = 330
SBP (mmHg)	114.5 ± 8.1	1.4 ± 4.4	14.4 ± 5.2 ‡	9.5 ± 7.7 || ◊
DBP (mmHg)	70.6 ± 7.4	-1.9 ± 3.8 †	11.4 ± 2.9 ‡	3.6 ± 5.2 # §
MAP (mmHg)	87.9 ± 7.5	-0.5 ± 4.1	12.7 ± 4.0 ‡	5.3 ± 4.7 || §
HR (bpm)	55.9 ± 7.5	2.3 ± 3.8 *	4.8 ± 6.9 ¶	-1.2 ± 6.5 ℓ

Data are mean ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP mean arterial pressure; HR, heart rate. * P<0.05, † P=0.06 for effect of hyperinsulinemia (hyperinsulinemia vs. basal insulin levels) in clamp study (*t* = 210 vs. *t* = 0). ‡ P<0.001, ¶ P=0.01 for effect of AngII infusion in clamp study (hyperinsulinemia + AngII vs. hyperinsulinemia) (*t* = 330 vs. *t* = 210). || P<0.001, # P<0.01 for effect of PE infusion in clamp study (hyperinsulinemia + PE vs. hyperinsulinemia) (*t* = 330 vs. *t* = 210). § P<0.001, ℓ P<0.01, ◊ P=0.03 for effect of AngII vs. effect of PE infusion in the clamp
study.

Effects of AngII and PE on insulin sensitivity (M/I value)

The clamp experiments with infusion of AngII and PE showed that normoglycemia was maintained during insulin, AngII, and PE infusion (5.0±0.4, 4.9±0.5, and 5.1±0.2 mmol/l, respectively). Insulin levels were elevated from 27.4±9.1 pmol/l to 429.9±74.1 pmol/l during the hyperinsulinemic clamp. M/I values were similar between the two clamp days [i.e. the mean difference in M/I value was 0.06±0.59 (mg/kg/min per pmol/l) × 100]. AngII and PE infusion resulted in a similar, significant increase in glucose uptake (M value in milligrams per kilogram per minute) (Table 3.5). However, PE, but not AngII, infusion significantly increased plasma insulin levels (P=0.02). Insulin sensitivity (M/I value) was thus augmented during AngII infusion but not during PE infusion (Table 3.5). In the 13 subjects with complete capillaroscopic data sets, insulin sensitivity data were similar (2.44±0.75 during hyperinsulinemia vs. 2.70±0.70 during AngII at hyperinsulinemia; P=0.065). Data from the 5.5h hyperinsulinemic time- control, without coinfusion of AngII or PE (n=8), showed that insulin sensitivity (M/I value) did not change over time. Approximately 60 min after insulin infusions were begun, plasma glucose, plasma insulin, and glucose infusion rates were at steady-state (data not shown). Insulin sensitivity (M/I value) was 2.40±0.68 at t = 150–210 min of hyperinsulinemia compared to 2.43±0.53 (mg/kg/min per pmol/liter) × 100 at t = 270–330 min of hyperinsulinemia (P=0.81).

Table 3.5. Metabolic variables during the hyperinsulinemic clamp (mean of two clamp days), AngII and PE infusion (N=18)

	Hyperinsulinemia *t* = 210	Hyperinsulinemia + AngII *t* = 330	Hyperinsulinemia + PE *t* = 330
Plasma glucose (mmol/l)	5.0 ± 0.4	4.9 ± 0.5	5.1 ± 0.2
Plasma insulin (pmol/l)	429.9 ± 74.1	445.5 ± 60.1	478.2 ± 99.2 ‡
M value (mg/kg/min)	10.1 ± 2.4	11.7 ± 2.4 *	11.5 ± 2.6 ¶
M/I value ((mg/kg/min per pmol/l)*100)	2.40 ± 0.71	2.68 ± 0.64 †	2.58 ± 1.1

Data are mean ± SD. * P<0.001, † P<0.01 Hyperinsulinemia + AngII vs. hyperinsulinemia. ‡ P=0.02, ¶ P<0.001 hyperinsulinemia + PE vs. hyperinsulinemia.

Discussion

This study, in healthy individuals, has three main findings. First, physiological hyperinsulinemia increases skin capillary densities at baseline, during reactive hyperemia-induced recruitment, and during venous congestion. Second, during physiological hyperinsulinemia, AngII decreases skin capillary densities both in absolute terms and compared with PE. Third, AngII is associated with an increase, not a decrease, in insulin-mediated glucose uptake. We conclude, therefore, that the effect of AngII to increase insulin-mediated glucose disposal in healthy volunteers is unlikely to be mediated by effects of AngII on hyperinsulinemia-induced increases in nutritive capillary density.

There is convincing evidence that insulin-induced changes in skeletal muscle microvascular blood flow distribution play an important role in regulating muscle glucose disposal.[16,17] In accordance, our data confirm previous findings that physiological hyperinsulinemia increases skin capillary densities at baseline and during reactive hyperemia (capillary recruitment) in healthy individuals.[22,26,28] A new finding is that hyperinsulinemia also increases capillary density during venous congestion, which is thought to expose all anatomically present capillaries.[31,32] The current data thus suggest that this concept may need revision and that it is not clear how the true maximal capillary density should be assessed. Regardless of this issue, it is the capillary density during reactive hyperemia (capillary recruitment) that is thought to be the most physiologically relevant variable, because it correlates closely with blood pressure and insulin-mediated glucose uptake[30] and because acute changes in capillary recruitment parallel acute changes in insulin-mediated glucose uptake.[20,22]

A key finding was that systemic AngII infusion augmented insulin-mediated glucose uptake while at the same time reducing insulin-stimulated capillary recruitment. Our finding that AngII increased whole-body glucose uptake in the presence of insulin is in agreement with previous studies in healthy individuals.[3,10–12] In these studies, the increases in whole-body glucose uptake (M value) due to AngII infusion (0.1–15 ng/kg/min) ranged

from 17–25%,[3,10–12] with larger effects found with higher doses of AngII. In this study, the increase in whole-body glucose uptake (M value) with AngII infusion was 16% and thus comparable to other studies. But what are the underlying mechanisms for increases in whole-body glucose uptake with AngII? In two studies, the AngII-mediated increase in muscle glucose utilization was attributed to concomitant increases in insulin-stimulated total muscle blood flow.[3,10] However, there is now substantial evidence that changes in total muscle flow do not affect insulin-stimulated glucose uptake.[17,21] Importantly, our data suggest that changes in nutritive capillary density also cannot explain the increase in insulin-mediated glucose uptake induced by AngII. Animal studies have provided evidence that AngII can increase nutritive perfusion,[19,24,33] possibly via the AngII type 2 receptor.[24] However, in contrast to our study, AngII was infused in the absence of insulin[19] or at fasting insulin levels.[24] Therefore, we suggest that AngII cannot further enhance nutritive capillary density under conditions of hyperinsulinemia, which itself is already a strong stimulus for nutritive capillary recruitment, even though it may do so at fasting insulin levels.

What other mechanisms might explain the effect of AngII to stimulate glucose disposal? First, insulin delivery depends not only on nutritive capillary perfusion, but also on net transendothelial transport of insulin, i.e. permeation.[34] Animal studies of single-vessel permeability indicated that AngII facilitates endothelial transport.[35,36] In addition, permeation depends not only on permeability but also on pressure and capillary surface area.[16,23] In this regard, although our data suggest that AngII decreases total capillary surface area, it is important to stress that we cannot exclude that AngII in fact increases capillary pressure and, potentially, permeation. Thus, the net effect of AngII on insulin delivery, and of vasopressors in general, cannot necessarily be predicted from their effects on capillary recruitment (as an estimate of capillary surface area). It follows, then, that there is no necessary contradiction in the fact that the effects of AngII and PE on capillary densities were not paralleled by similar effects on insulin-mediated glucose uptake. Second, AngII may affect insulin signaling (i.e. the phosphatidylinositol 3-kinase/Akt pathway) at the level of the muscle cell. However, most studies indicate that AngII impairs insulin signaling in metabolic and vascular tissues, likely due to AngII-induced reactive oxygen species production.[37,38] Furthermore, an animal study demonstrated that systemically infused AngII was very poorly taken up by skeletal muscle cells.[39] Therefore, it is unlikely that intravenously infused AngII in this study affected muscle cell insulin signaling. If AngII indeed does not affect muscle cell insulin signaling, it follows that the AngII-associated increase in insulin-mediated glucose uptake in healthy individuals must be due to increased delivery of glucose and insulin, possibly through mechanisms described above.

Importantly, in subjects with hypertension, obesity, or type 2 diabetes, conditions that are associated with a chronically up-regulated renin-angiotensin system (i.e. reflected in elevated circulating and tissue AngII levels),[40,41] the effect of AngII appears to be reversed,

because blocking the renin-angiotensin system with ACE inhibitors and angiotensin receptor blockers increases insulin-mediated glucose uptake in these subjects.[4–8] It is not clear how this can be explained, but taken together, these[6,7] and the current data suggest three possibilities. First, the effect of AngII to increase insulin delivery is preserved but is overruled by an AngII-induced impairment in muscle cell insulin signaling, resulting in a net negative effect of AngII on insulin-mediated glucose uptake; second, the effect of AngII to increase insulin delivery is itself reversed; and third, the effect of AngII on insulin delivery is diminished or reversed, while at the same time AngII induces an impairment in muscle cell insulin signaling. Clearly, these hypotheses need further testing, which will require studying the effects of AngII and of specific angiotensin type 1 and 2 receptor blockers under hyperinsulinemic conditions in healthy subjects and in subjects with hypertension, obesity, or type 2 diabetes.

The hemodynamic data in this study showed that, compared with basal insulin levels, hyperinsulinemia reduced the pressor response to PE, a peripherally acting α1-adrenoreceptor agonist, whereas it did not affect the pressor response to AngII. These findings are comparable to previous studies.[42,43] The explanation for this difference is not clear but might be related to AngII-induced stimulation of the sympathetic nervous system in addition to its direct effects on vascular tone. Thus, although coinfusion of a vasodilator (in this study insulin) with PE results in attenuation of the direct effects on vascular tone, coinfusion of a vasodilator with AngII may attenuate the effects of AngII on vascular tone but does not affect the AngII-mediated stimulation of the sympathetic nervous system.[44]

A possible limitation of the present study is that although muscle is the main peripheral site of insulin-induced microvascular function and insulin-mediated glucose uptake, we studied skin and not muscle microvascular function. However, the skin is the only site available in humans to examine capillary numbers directly, dynamically, and noninvasively. Importantly, there is substantial evidence that skin cutaneous microcirculation is a representative of microcirculation in general (e.g. skeletal muscle). This is underscored by several studies demonstrating that cutaneous microvascular function mirrors generalized systemic microvascular function.[25] With regard to insulin infusion, several studies have demonstrated comparable metabolic[45,46] and vascular effects[21–23,26] of insulin in muscle and skin. In addition, skin microvascular vasodilator capacity is associated with both insulin's vascular[30] and metabolic[30,47] actions in skeletal muscle. The latter is clearly demonstrated in a study in which acutely increasing or decreasing skin nutritive flow has been shown to result in parallel changes in skeletal muscle insulin-mediated glucose uptake.[22]

The present study, with a sample size of 13 subjects, was designed to have sufficient power to detect a significant increase in capillary recruitment and insulin sensitivity with AngII infusion. Despite this, we were not able to detect a significant increase in insulin sensitivity with AngII in 13 subjects (P=0.065), although the increase was numerically similar

to the increase found in 18 subjects (P<0.01). We chose to present insulin sensitivity data of 18 subjects because we know from the present data (n=18) and the existing literature[3,10–12] that AngII increases whole-body glucose. Restricting the insulin sensitivity data to n=13 would therefore lead to a false-negative finding (type 2 error).

In conclusion, our findings suggest that acute infusion of AngII is a potent stimulus for insulin-mediated whole-body glucose uptake. The mechanisms of the AngII-induced increase in whole-body glucose uptake are unclear, but the accelerated glucose uptake was probably not related to increases in microvascular perfusion because infusion of AngII, during hyperinsulinemia, reduced insulin-mediated skin capillary recruitment. Additional studies are required to fully understand the precise molecular mechanism of AngII-induced enhancement of insulin-mediated glucose uptake and the pathological changes in AngII-induced insulin resistance in hypertension and obesity.

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4

Acute angiotensin II receptor blockade improves insulin-induced microvascular function in hypertensive individuals

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Abstract

Background and aims - An effect of insulin that is crucial for stimulating glucose uptake is its ability to increase the number of perfused capillaries, and thereby enhance its own delivery, and that of glucose, to muscle cells. To unravel possible mechanisms involved in the insulin-sensitizing effects of angiotensin II receptor blockers (ARBs) in hypertensive individuals we investigated the effect of single-dose ARB administration on insulin-mediated microvascular perfusion in hypertensive individuals.

Methods - We examined the effects of ARB administration on hyperinsulinemia-associated capillary density by measuring baseline skin capillary density, capillary density during reactive hyperemia (hyperemic capillary recruitment), and capillary density during venous congestion in 17 hypertensive individuals in the basal state, during a hyperinsulinemic euglycemic clamp, and during a hyperinsulinemic clamp with acute ARB administration (600mg irbesartan), acute calcium channel blockade (CCB; 10mg felodipine ER), as a control for the reduction in blood pressure, or placebo. In addition, insulin sensitivity and blood pressure were measured.

Results - Compared to the basal state, hyperinsulinemia increased baseline capillary density (57.3±6.8 vs. 60.3±7.9 n/mm^2, P<0.01), but not hyperemic capillary recruitment. ARB and CCB treatment induced similar blood pressure reductions. Compared to placebo, ARB, but not CCB, increased hyperinsulinemia-associated baseline capillary density (+2.3±3.4 (P=0.02) and -0.4±4.4 n/mm^2, respectively). Hyperinsulinemia-associated hyperemic capillary recruitment was not altered by either treatment. Compared to placebo, neither ARB nor CCB treatment enhanced insulin sensitivity.

Conclusion - Acute ARB administration increases insulin-induced microvascular perfusion in mildly hypertensive individuals; this beneficial effect on microvascular perfusion was however not associated with increased insulin-mediated glucose uptake.

Introduction

A growing number of clinical trials have demonstrated that antihypertensive treatment with drugs that inhibit the renin-angiotensin system (RAS) reduces the risk for developing type 2 diabetes in hypertensive individuals.[1,2] The antidiabetogenic action of angiotensin II receptor blockers (ARBs) has been largely attributed to improvements in peripheral insulin sensitivity.[3-7] However, although several potential mechanisms have been proposed for these insulin-sensitizing effects of ARBs (e.g. adipose tissue modification,[8] reduced sympathetic activity,[9] activation of peroxisome proliferator-activated receptor gamma,[10] and reduced oxidative stress[11]) so far, the exact mechanisms, at molecular and organ systems levels, have not been fully explained.

It has become clear that, in addition to muscle cells, the vasculature is an important physiological target for insulin.[12,13] Insulin acts at key sites in the vasculature of muscle to increase the number of perfused capillaries. This insulin-induced increase in microvascular perfusion is mediated via activation of the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway and the production of nitric oxide (NO) in the vascular endothelium,[14] and has been shown to be a significant regulator of overall insulin-mediated glucose uptake.[15,16] Since there is substantial evidence for a physiologically relevant crosstalk between the insulin and RAS signaling pathways,[17,18] we hypothesized that an improvement in the ability of insulin to increase the number of perfused capillaries may be a possible mechanism contributing to the metabolic benefits of ARBs.

Skeletal muscle precapillary arterioles are a major site of RAS action in vivo[19] and *in vitro* studies have repeatedly shown that angiotensin II (AngII) impairs vascular insulin signaling and reduces insulin-stimulated NO production.[18,20] In addition, *ex vivo* studies demonstrated impaired vascular insulin PI3-kinase/Akt signaling and impaired insulin-stimulated NO-dependent vasodilation in different animal models of hypertension.[11,21,22] Therefore, we specifically hypothesized that chronic overactivity of the RAS in hypertension leads to an impaired action of insulin to increase the number of capillaries perfused (i.e. microvascular insulin resistance) and consequently to the development of metabolic insulin resistance. Conversely, we hypothesized that ARB treatment will improve the capillary responses to insulin and, subsequently, insulin-mediated glucose uptake in hypertensive individuals. To date, the effects of ARBs on microvascular perfusion during hyperinsulinemia have not been studied.

Therefore, in the present study we examined the effects of single-dose ARB administration on insulin-induced microvascular perfusion and insulin-stimulated glucose uptake in hypertensive individuals.

Subjects and methods

Subjects

Seventeen non-obese (body mass index <30kg/m^2) individuals with essential hypertension participated in this study. All subjects were Caucasian, non-smokers, non-diabetic, as defined by fasting plasma glucose levels <7.0 mmol/l,[23] and had no signs or symptoms of cardiovascular or other concomitant disease. Fifteen subjects were taking antihypertensive medication at the time of inclusion. Six of these were treated with an ARB alone (n= 1), or in combination with a β-blocker (n=1), a calcium channel blocker (CCB; n = 2), a thiazide diuretic (n=1), or a β-blocker with a CCB (n=1); seven subjects were treated with angiotensin-converting enzyme inhibitors alone (n=2), or in combination with a thiazide diuretic (n=3), or a CCB with (n=1), or without a diuretic (n=1); two subjects were treated with CCBs in combination with a β-blocker (n=1) or in combination with a diuretic (n=1). All antihypertensive medication was discontinued three weeks before the studies. Thereafter all subjects underwent a 24h ambulatory blood pressure measurement (ABPM). The average daytime blood pressure levels of all subjects were >135/85 mmHg. None of the participants used any medication during the studies. All participants gave informed consent for the study. The study was approved by the institutional review committee and performed in accordance with the Declaration of Helsinki.

Study design

All individuals were allocated to three interventions in random order according to a double-blind design, i.e. a euglycemic hyperinsulinemic clamp with administration of a single oral dose of 600 mg irbesartan (ARB); a euglycemic hyperinsulinemic clamp with administration of a single oral dose of 10 mg felodipine ER (CCB), as a control for the reduction in blood pressure; or a euglycemic hyperinsulinemic clamp with placebo administration, as a hyperinsulinemic time-control (Figure 4.1). Irbesartan was chosen as it is a highly specific competitive antagonist of AngII type 1 receptors with a high receptor affinity and a rapid onset of action.[24] A high dose was chosen in order to obtain maximal AngII type 1 receptor blockade. Indeed, before conducting these experiments, we confirmed, in six normotensive healthy individuals, that this dose was sufficient to fully block the response to exogenous AngII. Felodipine was chosen as a comparator for the vasodilator effects of irbesartan, as CCBs are considered to have a neutral effect on insulin sensitivity.[2] In the same six individuals we confirmed that treatment with 600 mg irbesartan and 10 mg felodipine ER resulted in identical blood pressure reductions. The interval between each of the three study days was one week. In order to standardize the activity of the endogenous RAS, subjects adhered to a moderately sodium restricted diet (100 mmol/day) for one week prior to the first visit, and also prior to the following visits. Compliance with the diet was assessed by two measurements of 24h urinary sodium excretion.

All measurements were conducted in a quiet, temperature-controlled room (T=23.4±0.5°C) at 8.00 a.m., after a 10h fast, with the subjects in the supine position. Subjects were asked to refrain from drinking alcohol for a period of 24h before each study day and to perform no strenuous exercise for a period of 48h before each study day. Baseline measurements were obtained after allowing 30 min of rest and acclimatization after insertion of two i.v. catheters (Venflon, Viggo, Gotenborg, Sweden). Skin temperature was monitored during the tests.

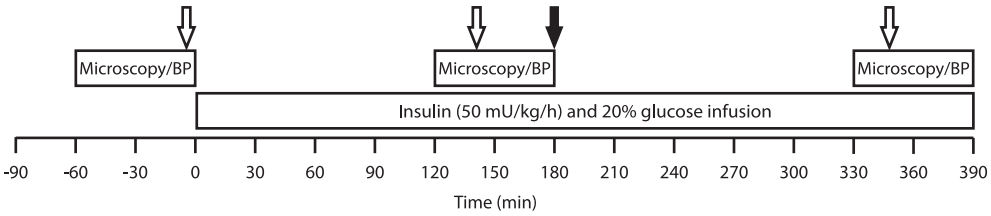

Figure 4.1. Design of the study. There were three interventions: hyperinsulinemic clamp with single-dose ARB administration, hyperinsulinemic clamp with single-dose CCB administration, and hyperinsulinemic clamp with placebo administration. Microscopy indicates capillary microscopy of the skin of the finger; BP, blood pressure measurements; ⇩, blood samples for measurements of insulin concentrations; ⬇, intake of ARB, CCB or placebo. Baseline microvascular measurements (t = –60–0 min) were performed on only one of the experimental days (randomly assigned). Microvascular measurements during the hyperinsulinemic clamp and during clamp plus drug administration were performed during all three study days.

Hyperinsulinemic euglycemic clamp

Insulin sensitivity was assessed by the hyperinsulinemic euglycemic clamp method, using a modification of the method described by DeFronzo.[25] Briefly, insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was infused in a primed continuous manner at a rate of 50mU/kg/h. Mean fasting glucose concentrations were determined from three glucose concentrations measured before t = 0 min. Normoglycemia was maintained by adjusting the rate of a 20% D-glucose infusion based on plasma glucose measurements performed at 5-10 min intervals. Whole-body glucose uptake (M) was calculated from the glucose infusion rates during the last 60 min of hyperinsulinemia (t = 120-180 min) and the last 60 min of hyperinsulinemia plus ARB, CCB and placebo administration (t = 330-390 min). M was expressed per kg body weight per unit of plasma insulin concentration multiplied by 100 (M/I), thereby correcting for differences in steady-state insulin concentrations.[26] Insulin concentrations during the hyperinsulinemic clamp were determined from steady-state insulin levels measured at t = 140 min. Insulin concentrations during hyperinsulinemia with ARB, CCB and placebo administration were determined from steady-state insulin levels measured at t = 350 min.

Capillary microscopy

Nailfold capillary studies were performed at baseline (*t* = -60-0 min; on one study day), during hyperinsulinemia (*t* = 120-180 min; on three study days) and during hyperinsulinemia with ARB, CCB or placebo intake (*t* = 330-390 min). Nailfold capillaries in the dorsal skin of the fourth finger were visualized by a capillary microscope (Leitz-Orthoplan) in combination with a CCD-camera. To visualize the capillaries, a 4.0 objective (Leitz N.A. 0.14) was used with a total system magnification of 160x. Capillaries were visualized ~4.5 mm proximal to the terminal row of capillaries in the middle of the nailfold. Subsequently, a characteristic capillary was kept on the same spot of the visual field (marked by a dot on the monitor) to ensure that capillary density was measured in the exact same visual field during the entire experiment. Baseline capillary density was defined as the number of continuously erythrocyte-perfused capillaries per square millimeter of nailfold skin. Postocclusive reactive hyperemia (PRH) after 4 min of arterial occlusion was used to assess the functional reserve capacity (hyperemic capillary recruitment). The number of capillaries at baseline and directly after release of the cuff were counted for, respectively, 30 and 45 s from a freeze-framed reproduction of the videotape and from the running videotape when it was uncertain whether a capillary was present or not. Capillaries were counted offline by a single experienced investigator who was blinded to the experimental status of the recordings. In addition, we applied venous congestion, with the digital cuff inflated to 60 mmHg for 60 s, to expose a maximal number of capillaries.[27] Capillaries during venous congestion were counted in the 60-second recordings. All procedures were performed on two separate visual fields of 1 mm^2 and the mean of both measurements was used for analyses. The day-to-day coefficients of variation (CV) of baseline capillary density, hyperemic capillary recruitment and number of capillaries during venous congestion during hyperinsulinemia were 6.4, 6.9, and 4.2%, as determined in 13 individuals on two separate days.

Blood pressure

Ambulatory monitoring (SpaceLabs 90207, Redmond, WA, USA) was used to obtain 24h recordings of blood pressure and heart rate. The non-dominant arm was used with an appropriately sized cuff. During study days, blood pressure and heart rate were determined at the times specified in Figure 4.1 by means of an automatic device (Datascope Accutorr Plus™, Paramus, NJ, USA). The mean of the second and third measurement was used for further analyses.

Biochemical measurements

Plasma insulin concentrations were measured by radioimmunoassay technique (AutoDELFIA, PerkinElmer, Massachusetts, USA). Blood glucose concentrations were determined with a glucose analyser YSI2300 (Yellow Springs Instrument, Yellow Springs,

OH, USA).

Statistical analysis

Data are expressed as mean ± SD. First, a paired Student's *t* test was used to study effects of hyperinsulinemia, CCB, ARB, and placebo on skin microvascular function within experimental days. Second, a paired Student's *t* test was used to compare the effects of ARB and placebo on insulin-associated microvascular function and to compare the effects of CCB and placebo on insulin-associated microvascular function.

A paired Student's *t* test was used to study effects, versus placebo, of hyperinsulinemia, ARB and CCB treatment on blood pressure and to study the effects of ARB and CCB administration on insulin sensitivity (M/I value). A two-tailed P value of <0.05 was considered significant. All analyses were performed using the statistical software package SPSS version 15.0.

Results

Subjects

The participants were middle-aged, mildly overweight individuals with grade I or grade II hypertension and normal fasting glucose levels (Table 4.1). Individuals were insulin resistant as compared to healthy normotensive individuals included in a previous study[28] with a comparable clamp protocol (M/I value: 1.28±0.51 vs. 2.40±0.71 (mg/kg/min per pmol/l)*100)). The urinary sodium excretion data showed adequate compliance with the moderately sodium-restricted diet (Table 4.1).

Table 4.1. Characteristics of the study population

Number (men/women)	17 (11/6)
Age (years)	52.2 ± 5.7
Body mass index (kg/m^2)	26.7 ± 2.2
Waist-to-hip ratio (men)	0.93 ± 0.06
Waist-to-hip ratio (women)	0.81 ± 0.04
SBP daytime, 24h-ABPM (mmHg)	149 ± 12
DBP daytime, 24h-ABPM (mmHg)	96 ± 6
Fasting plasma glucose (mmol/l)	5.2 ± 0.4
Fasting insulin (pmol/l)	51.5 ± 26.6
Insulin sensitivity (M/I value)	1.28 ± 0.51
Urinary sodium excretion (mmol/24h)	106.7 ± 59.8

Values are expressed as mean ± SD, or number. ABPM, ambulatory blood pressure monitoring. M/I value is expressed in (mg/kg/min per pmol/l) × 100.

Effects of acute ARB and CCB administration on blood pressure and heart rate during hyperinsulinemia (Table 4.2)

Hemodynamic variables during the studies are shown in Table 4.2. Hyperinsulinemia

induced a significant decrease in DBP. During hyperinsulinemia, placebo administration did not induce a further effect on blood pressure and heart rate. Compared to placebo, acute ARB and CCB administration markedly decreased MAP, SBP, and DBP during hyperinsulinemia, while CCB, but not ARB administration, significantly increased heartrate during hyperinsulinemia. The effects of ARB and CCB administration on MAP, SBP, and DBP during hyperinsulinemia were comparable. The increase in HR with CCB administration during hyperinsulinemia was significantly different from that of ARB (Table 4.2; P<0.001 for the change with ARB vs. the change with CCB).

Table 4.2. Hemodynamic changes during the hyperinsulinemic clamp (mean values of three clamp days) and subsequent ARB, CCB, and placebo administration

	Basal state *t* = 0	Hyperinsulinemia *t* = 180	Δ ARB - hyperinsulinemia *t* = 180-390	Δ CCB - hyperinsulinemia *t* = 180-390	Δ Placebo - hyperinsulinemia *t* = 180-390
SBP (mmHg)	139 ± 11	140 ± 11	-6.5 ± 7.7 ‡	-5.5 ± 7.9 ‖	+0.2 ± 7.3
DBP (mmHg)	89 ± 7	86 ± 7 *	-5.2 ± 3.7 †	-5.6 ± 4.9 §	+0.7 ± 3.9
MAP (mmHg)	109 ± 8	109 ± 9	-7.3 ± 5.1 †	-8.2 ± 6.0 §	-0.5 ± 5.6
HR (bpm)	61 ± 6	60 ± 6	+1.7 ± 2.8 ¶	+7.4 ± 4.4 §	+1.4 ± 3.5

Values are expressed as mean ± SD. *P<0.001 for effect of hyperinsulinemia (hyperinsulinemia vs. basal state). †P=0.001; ‡P=0.02 for effect of ARB vs. effect of placebo during hyperinsulinemia (Δ ARB-hyperinsulinemia vs. Δ Placebo-hyperinsulinemia). §P<0.01, ‖P<0.05 for effect of CCB vs. effect of placebo during hyperinsulinemia (Δ CCB-hyperinsulinemia vs. Δ Placebo-hyperinsulinemia). ¶P<0.001 for effect of ARB vs. effect of CCB during hyperinsulinemia (Δ ARB-hyperinsulinemia vs. Δ CCB-hyperinsulinemia).

Effects of insulin on capillary density (Table 4.3)

Due to technical difficulties with the videomicroscopy we were unable to evaluate the effects of hyperinsulinemia on baseline skin microvascular function and the effects of CCB and placebo on insulin-associated microvascular function in one subject, and to evaluate the effects of ARB treatment in two subjects.

Hyperinsulinemia significantly increased baseline capillary density, but not hyperemic capillary recruitment or density during venous congestion (Table 4.3). In previous work we have demonstrated that this is not a time- and/or volume-dependent effect.[28,29]

Table 4.3. Nailfold capillary densities before and during the hyperinsulinemic clamp

	Basal state *t* = 0	Hyperinsulinemia *t* = 180
Baseline density (n/mm^2)	57.3 ± 6.8	60.3 ± 7.9 *
Capillary recruitment PRH (n/mm^2)	67.8 ± 9.9	69.1 ± 10.7
Venous congestion (n/mm^2)	75.8 ± 10.8	76.9 ± 11.6

Values are expressed as mean ± SD. n/mm^2, number of perfused capillaries per mm^2. PRH, peak reactive hyperemia. *P<0.01 vs. basal state.

Effects of acute ARB and CCB administration on capillary density during hyperinsulinemia (Figure 4.2)
Acute ARB, and not CCB administration, significantly increased hyperinsulinemia-stimulated baseline capillary density, while placebo administration significantly decreased hyperinsulinemia-stimulated baseline capillary density. The increase in hyperinsulinemia-stimulated baseline capillary density with ARB was significantly different from the decrease with placebo (P=0.02; Figure 4.2). Neither ARB, nor CCB or placebo induced a significant effect on hyperinsulinemia-associated hyperemic capillary recruitment. ARB and placebo administration did not significantly affect hyperinsulinemia-associated capillary density during venous congestion, while CCB administration significantly increased this variable. This increase in hyperinsulinemia-associated capillary density during venous congestion with CCB administration was significantly different from the non-significant decrease with placebo treatment (P=0.001; Figure 4.2). Skin temperature was not significantly different during the three hyperinsulinemic clamps (mean temperature 30.3±2.1°C). The mean increase in skin temperature with drug administration was +0.8±1.8°C and was comparable between the drugs.

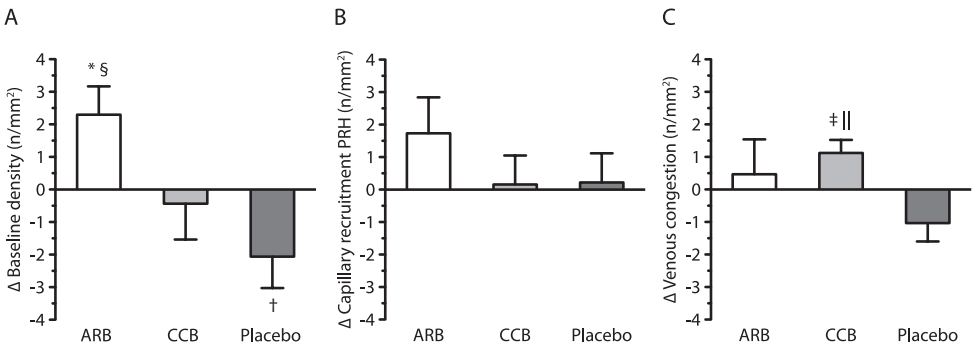

Figure 4.2. Changes in baseline capillary density (A), hyperemic capillary recruitment (B), and density during venous congestion (C) due to ARB, CCB, and placebo administration during hyperinsulinemia. Each bar represents mean±SD. *P=0.02 for the increase in baseline density with ARB administration during hyperinsulinemia, †P<0.05 for the decrease in baseline density with placebo administration during hyperinsulinemia, ‡P=0.01 for the increase in density during venous congestion with CCB administration during hyperinsulinemia. §P=0.02 for the effect of ARB vs. the effect of placebo during hyperinsulinemia (ARB-hyperinsulinemia vs. Placebo-hyperinsulinemia), ||P=0.001 for the effect of CCB vs. the effect of placebo during hyperinsulinemia (ARB-hyperinsulinemia vs. CCB-hyperinsulinemia).

Effects of acute ARB and CCB administration on insulin sensitivity (M/I value)
Table 4.4 shows the metabolic variables during the three hyperinsulinemic euglycemic clamps before (*t* = 180 min) and after ARB, CCB or placebo administration (*t* = 390 min). Normoglycemia was maintained during hyperinsulinemia and during hyperinsulinemia with ARB, CCB, and placebo administration. Plasma insulin levels, M values, and M/I values did not differ between the three hyperinsulinemic euglycemic clamps before drug

administration.

Compared to hyperinsulinemia before drug administration, CCB, but not ARB or placebo, significantly decreased plasma insulin levels (Table 4.4). Compared to hyperinsulinemia before drug administration, both ARB and CCB significantly increased glucose uptake (M value) and insulin sensitivity (M/I value). However, glucose uptake (M value) and insulin sensitivity (M/I value) were also significantly increased by placebo administration (i.e. prolonged levels of hyperinsulinemia). The increases in glucose uptake (M value) and insulin sensitivity (M/I value) with ARB and CCB administration were not significantly different from the increases in M value and M/I value with placebo (Table 4.4).

Discussion

This study, in mildly hypertensive individuals, has three main findings. First, physiological hyperinsulinemia increases baseline skin capillary density. Second, during physiological hyperinsulinemia, acute treatment with the ARB, irbesartan, but not the CCB, felodipine, further increases skin baseline capillary density. Third, acute ARB treatment is not associated with an increase in insulin-mediated glucose uptake. We conclude, therefore, that acute ARB treatment positively affects capillary density in hypertensive individuals during hyperinsulinemia, but that this improved microvascular function is not associated with increased insulin-mediated glucose uptake.

There is convincing evidence that insulin-induced changes in skeletal muscle microvascular blood flow distribution play an important role in regulating muscle glucose disposal.[12,13] Accordingly, previous studies, in healthy individuals, have demonstrated that physiological hyperinsulinemia increases capillary densities at baseline and during reactive hyperemia in both skin and skeletal muscle.[28-31] Our data demonstrate that physiological hyperinsulinemia also increases baseline capillary density in hypertensive individuals. In addition, acute ARB administration further augmented baseline capillary density.

The increase in insulin-induced microvascular perfusion found with ARB treatment in this study is in agreement with a study in salt-sensitive hypertensive rats, in which deterioration of insulin-mediated vasorelaxation induced by high-salt feeding was prevented by six weeks of ARB treatment.[11] However, in the animal study insulin-induced vasorelaxation was measured in aortic rings and not further down the arterial tree at (pre)capillary levels, where microvascular perfusion and insulin extraction into skeletal muscle are regulated.[12] The only study, in rats, performed on the effects of ARB treatment on skeletal muscle microvascular perfusion demonstrated a threefold increase in microvascular perfusion in the presence of losartan.[19] However, in this study, losartan was infused in normotensive rats at fasting insulin levels,[19] while most glucose uptake takes place postprandially, i.e. during hyperinsulinemia. Thus, in the present study, we demonstrated for the first time that acute ARB treatment increased functional capillary density during hyperinsulinemia in

Table 4.4. Metabolic variables during the hyperinsulinemic clamp and subsequent administration of ARB, CCB, and placebo

	Hyperin-sulinemia pre ARB *t* =180	Hyperin-sulinemia + ARB *t* = 390	Δ ARB-hyperin-sulinemia *t* =180-390	Hyperin-sulinemia pre CCB *t* = 180	Hyperin-sulinemia + CCB *t* = 390	Δ CCB-hyperin-sulinemia *t* = 180-390	Hyperin-sulinemia pre placebo *t* = 180	Hyperin-sulinemia + placebo *t* = 390	Δ Placebo-hyperin-sulinemia *t* = 180-390
Plasma glucose (mmol/l)	5.18 ± 0.48	5.24 ± 0.54	+0.06 ± 0.23	5.09 ± 0.54	5.11 ± 0.44	+0.09 ± 0.24	5.16 ± 0.23	5.16 ± 0.35	-0.00 ± 0.21
Plasma insulin (pmol/l)	555.1 ± 142.7	558.0 ± 142.6	+4.4 ± 51.7	568.8 ± 118.1	515.4 ± 140.0 *	-62.6 ± 47.8 §	544.1 ± 123.1	549.1 ± 143.6	+5.0 ± 58.2
M value (mg/kg/min)	6.49 ± 2.01	8.11 ± 2.35 †	+1.62 ± 1.46	6.97 ± 2.71	8.44 ± 2.97 ‡	+1.33 ± 1.63	6.63 ± 2.19	8.69 ± 2.50 †	+2.07 ± 1.40
M/I value	1.26 ± 0.52	1.56 ± 0.67 ‡	+0.31 ± 0.36	1.30 ± 0.63	1.83 ± 0.92 †	+0.49 ± 0.45	1.28 ± 0.51	1.69 ± 0.63 †	+0.40 ± 0.31

Values are expressed as mean ± SD. M/I value expressed in (mg/kg/min per pmol/l)*100. Δ represent metabolic changes due to ARB, CCB, and placebo administration during hyperinsulinemia. *P<0.001 for the decrease in plasma insulin level with CCB administration during hyperinsulinemia. †P<0.001, ‡P<0.01 for the increase in M value and M/I value with ARB, CCB, and placebo administration during hyperinsulinemia. §P<0.02 for the effect of CCB vs. the effect of ARB and the effect of placebo during hyperinsulinemia.

hypertensive individuals.

What might be the underlying mechanisms for this increase in insulin-associated microvascular density with ARB administration? In the present study, the increase in capillary density with ARB could not be attributed to blood pressure reduction since treatment with felodipine resulted in an identical blood pressure reduction, and no effect of felodipine on insulin-mediated baseline capillary density was found. Therefore, and as also indicated by our previous work,[28] these data suggest that the RAS has specific effects on insulin-induced microvascular function. Indeed, experimental studies have demonstrated that AngII inhibits insulin activation of the PI3-kinase/Akt pathway in vascular as well as skeletal muscle cells, probably via an increase in reactive oxygen species (ROS) and proinflammatory molecule expression.[11,17,18] This selective impairment of insulin activation of the PI3-kinase/Akt pathway is accompanied by activation of the insulin ERK 1/2 pathway, which is involved in the production of the vasoconstrictor endothelin-1.[14] As a result, the balance between the vasodilatory and vasoconstrictive properties of insulin is impaired and insulin becomes a vasoconstrictor of muscle precapillary arterioles.[14] Indeed, we have previously demonstrated that AngII infusion decreased the number of perfused capillaries during hyperinsulinemia in healthy individuals.[28] Thus, AngII has been shown to interfere with insulin signaling pathways and, in accordance, inhibition of the RAS has been shown to improve vascular insulin signaling in hypertensive rats.[11] Therefore, we suggest that the increase in hyperinsulinemia-associated capillary density with ARB treatment in this study is likely due to improved vascular insulin signaling and consequently increased insulin-induced NO production.

Although muscle microvascular perfusion is a determinant of insulin-mediated glucose uptake and acute ARB administration increased the number of perfused capillaries during hyperinsulinemia in this study, no increase in insulin-mediated glucose uptake was found. How can the absence of an increase of glucose disposal with increased capillary density be explained? First, small increases in microvascular perfusion may not be sufficient to increase insulin-mediated glucose disposal, as was shown in an elegant study of the dose-dependence of the microvascular and the metabolic effects of insulin, in which insulin increased the number of perfused capillaries at concentrations lower than those required to stimulate glucose disposal.[32] Second, the net effect of ARB treatment on insulin and glucose delivery cannot necessarily be predicted from its effects on the number of capillaries perfused. In fact, insulin and glucose delivery not only depend on capillary perfusion, but also on net transendothelial transport, i.e. permeation.[12] Permeation depends on permeability, but also on pressure and capillary surface area (i.e. in this study estimated as the number of capillaries perfused).[12] In this regard, although our data suggest that ARB treatment increases total capillary surface area, it is important to stress that we cannot exclude that ARB treatment in fact decreases capillary pressure

and, potentially, permeation. It follows, then, that there is no necessary contradiction in the fact that the effects of acute ARB treatment on capillary densities were not paralleled by similar effects on insulin-mediated glucose uptake. Third, the lack of an increase of glucose disposal with augmented microvascular perfusion might indicate that microvascular perfusion is not rate limiting for glucose disposal in hypertensive individuals. This would be in accordance with known capillary/tissue glucose exchange principles, which suggest that insulin-induced increases in capillary perfusion become rate limiting for glucose uptake in situations in which tissue permeability to glucose is high.[33] In contrast, when tissue permeability is relatively low, one would not expect increased capillary density to have as great a modulating effect on glucose uptake. In the latter situation, cellular permeability of glucose would be rate limiting for overall glucose uptake. Insulin resistant states, like hypertension, which are characterized by defects in insulin signaling impairing glucose transport,[34] would not be expected therefore to exhibit capillary perfusion-limited glucose exchange. Nevertheless, it should be appreciated that these findings by no means negate an important role of impaired microvascular insulin responses in the pathogenesis of insulin resistance,[35] as in individuals in whom cellular insulin action has been ameliorated, perfusion would be predicted to become relatively more rate limiting. It follows logically that complete normalization of insulin action requires a physiologic vascular response to insulin. Fourth, the duration of ARB treatment may be important to induce an effect on glucose disposal. Several studies have shown improved insulin-mediated glucose uptake with long-term ARB treatment,[3-7] while, in accordance with our study, the only other study performed with acute ARB treatment did not show an effect on glucose disposal.[36] The effect of chronic ARB treatment may be due to adaptive responses in the skeletal muscle glucose transport system independently of potential hemodynamic effects. Indeed, chronic treatment of hypertensive and obese Zucker rats with the ARBs olmesartan and irbesartan has been shown to restore skeletal muscle PI3-kinase/Akt insulin signaling.[34,37] Since delivery becomes rate limiting for glucose uptake in situations in which tissue permeability to glucose is high[33], the insulin-sensitizing effects of chronic ARB treatment may be explained by both direct effects on glucose transport and insulin signaling in skeletal muscle, and by microvascular effects.

Taken together, our findings demonstrate that acute ARB administration improves the capillary responses to insulin in mildly hypertensive individuals. Although this improvement in insulin-induced microvascular perfusion was not reflected in increased glucose disposal, more pronounced effects of ARB treatment on insulin's microvascular as well as metabolic functions may occur in a longer-term perspective.

Methodological differences may explain why, in this study, hyperinsulinemia as well as ARB administration affected baseline capillary density, while in previous studies we primarily reported effects on hyperemic capillary recruitment.[29,30] In previous studies we

determined baseline capillary density during a 15-second count at a magnification of 99x[27,29], whereas in the present study, due technical requirements of the microscopic device, we determined baseline capillary density during a 30-second count at a magnification of 160x. As a consequence, baseline capillary density thus obtained is considerably greater, and, conversely, baseline capillary density resembles hyperemic recruitment more closely, than in previous studies.[27,29,38]

During physiological hyperinsulinemia, acute CCB administration augmented capillary density during venous congestion, while the effect of acute ARB did not reach statistical significance (P=0.16). Venous congestion increases venous back pressure, which allows the passive trapping of red cells in nonperfused and intermittently perfused capillaries, increasing the number of red blood cell-filled capillaries. In addition, in order to maintain capillary pressure, venous congestion also induces arteriolar constriction via a venoarteriolar response. CCBs may impair this response.[39] The increase in capillary density during venous congestion might thus be related to arteriolar vasodilation (i.e. increased arteriolar influx and thus increased passive trapping of red cells) induced by both the ARB and the CCB, with a stronger arteriolar vasodilation for the CCB. An alternative interpretation is that the non-significant effect of ARB administration is a false negative finding (type 2 error) and that this increase in capillary density during venous congestion generally has to do with vasodilation.

A possible limitation of the present study is that although muscle is the main peripheral site of insulin-induced microvascular function and insulin-mediated glucose uptake, we studied skin and not muscle microvascular function. However, the skin is the only site available in humans to examine capillary numbers directly, dynamically and noninvasively. Importantly, there is substantial evidence that cutaneous microcirculation is a representative of microcirculation in general (e.g. skeletal muscle). This is underscored by several studies demonstrating that cutaneous microvascular function mirrors generalized systemic microvascular function.[40] With regard to insulin infusion, several studies have demonstrated comparable metabolic[41] and vascular effects[28,29,31] of insulin in muscle and skin. In addition, skin microvascular vasodilator capacity is associated with both insulin's vascular and metabolic actions in skeletal muscle.[27] The latter is clearly demonstrated in a study in which acutely increasing or decreasing skin nutritive flow has been shown to result in parallel changes in skeletal muscle insulin-mediated glucose uptake.[29]

In conclusion, the present study showed that acute ARB administration improves the capillary responses to insulin in mildly hypertensive individuals. This improved microvascular response to insulin was specific for RAS inhibition and is likely due to restoration of impaired vascular insulin signaling induced by chronic RAS overactivity in hypertensive individuals. Although this improvement in insulin-induced microvascular perfusion with acute ARB treatment was not reflected in an increase in insulin-mediated

glucose uptake, these observations may provide a new mechanism underlying the insulin-sensitizing effects of long-term ARB treatment. From a clinical perspective, the finding that insulin-mediated changes in capillary perfusion can be modified by ARB treatment might indicate that capillary function could represent a new therapeutic target aimed at blocking the progression and the clinical outcomes of insulin resistance. However, further, long-term, studies are required to determine whether normalized capillary responses to insulin can prevent the progression of insulin resistance to type 2 diabetes in hypertensive individuals.

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5

Meal-related increases in microvascular vasomotion are impaired in obese individuals: a potential mechanism in the pathogenesis of obesity-related insulin resistance

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Abstract

This article reviews and adds original data to the evidence for microvascular dysfunction, including impairment of insulin-stimulated microvascular perfusion as a key element in the pathogensis of obesity-related hypertension and insulin resistance.

Current knowledge in the field of insulin-stimulated microvascular perfusion is based mostly on steady-state hyperinsulinemia (i.e. as obtained with the hyperinsulinemic euglycemic clamp technique). Steady-state hyperinsulinemia has been shown to stimulate endothelium-dependent vasomotion, which contributes to increased glucose uptake; this phenomenon has been shown to be blunted in obesity. However, physiologically hyperinsulinemia is usually transient and dynamic. To study the effects of transient and dynamic hyperinsulinemia on microcirculatory function in regulating insulin-mediated glucose uptake, we performed a randomised, placebo-controlled trial in 18 lean and 13 obese individuals to examine the effects of a glucose drink, a liquid mixed meal, and tap water (control) on microvascular vasomotion. We found that meal ingestion increases microvascular vasomotion in healthy lean individuals and that this response is impaired in obesity. The lack of meal-induced stimulation of microvascular vasomotion in obese individuals paralleled blunted insulin-stimulated glucose disposal after the meal, which is consistent with a role for insulin-stimulated microvascular vasomotion in insulin-mediated glucose uptake in the postprandial state. Therefore, these data, under physiological conditions, support current evidence that impaired (insulin-induced) microvascular function in obese individuals is a possible mechanism in the pathogenesis of obesity-related insulin resistance.

Introduction

Obesity is associated with insulin resistance, hypertension, cardiovascular disease and type 2 diabetes[1], but the mechanisms underlying these associations are incompletely understood. This article reviews and adds original data (i.e. in the postprandial state) to the evidence for microvascular dysfunction, including impairment of insulin-stimulated microvascular perfusion as a key element in the pathogenesis of obesity-related hypertension and insulin resistance.[2,3]

Microvascular dysfunction contributes to high blood pressure and impairment of insulin-mediated glucose uptake

The microcirculation is widely taken to encompass vessels <150µm (i.e. arterioles, capillaries, and venules),[4] and has two important functions. First, arterioles regulate hydrostatic pressure and peripheral vascular resistance.[4] Importantly, dysfunction of the microcirculation, with concomitant increases in vasoconstrictor tone, will increase total peripheral resistance and, other things being equal, blood pressure, as reviewed elsewhere.[2,3] Second, it regulates tissue perfusion to optimize the delivery of nutrients and removal of waste products within tissues in response to variations in demand. In this respect, insulin has been shown to play an important role.[5]

Insulin redirects blood flow within the muscle microvascular bed to increase available capillary surface area, an effect referred to as capillary recruitment.[6,7] In addition, insulin induces vasodilation of resistance vessels resulting in an increase in total muscle blood flow.[8] Whether this increase in total muscle blood flow, which occurs later in time compared to the redirection of flow to nutritive capillary beds, serves to enhance insulin-mediated glucose uptake remains controversial.[9] However, it has been generally accepted that capillary recruitment is crucial for the delivery of insulin and glucose to tissue. Indeed, several studies have shown that insulin-mediated increases in capillary recruitment account for approximately half of insulin-mediated muscle glucose uptake in vivo.[10-14] Insulin's effect on microvascular blood flow is therefore an important regulator of insulin-mediated muscle glucose uptake, the main determinant of whole-body insulin sensitivity. Conversely, impairment of insulin-mediated increases in capillary recruitment will decrease insulin-mediated glucose uptake.[5]

Insulin-induced capillary recruitment is mediated via a vasodilatory action of insulin on precapillary muscle arterioles.[6,7] This action is critically dependent on insulin-stimulated production of nitric oxide (NO) in the vascular endothelium, an effect mediated by phosphatidylinositol 3-kinase (PI3-kinase), protein kinase B/Akt, and endothelial NO synthase (eNOS).[15,16] In fact, under basal conditions (i.e. at rest or in the preprandial state) only one-half to one-third of the capillary bed is perfused at any time[7,17] and these capillaries, moreover, are mainly shorter so-called non-nutritive capillaries.[18] By relaxation

of terminal arterioles connected to nutritive capillary networks (i.e. capillaries that perfuse sites of high glucose uptake), insulin redirects blood flow from relatively non-nutritive vessels to nutritive capillary beds, with a resultant increase in the overall number of perfused capillaries.[6] In addition to this NO-dependent vasodilator effect, insulin increases the secretion of the vasoconstrictor peptide endothelin-1 (ET-1)[15,16] via the intracellular MAP-kinase signaling pathway and the extracellular signal-regulated kinase-1/2 (ERK1/2).[19] In healthy individuals, there is a balance between the insulin-mediated NO- and ET-1-dependent effects, with the net effect being either neutral or vasodilatory.

Under normal conditions, arterioles are not "open" or closed" for any prolonged period of time. In fact, it is the rhythmic contraction and dilatation of arterioles, so-called vasomotion, which regulates microvascular flow distribution so that different muscle regions are intermittently perfused.[7,17] Capillary recruitment can thus be seen as a process wherein the total "open" time of capillary networks increases in conditions of increased metabolic demand. An experimental study has demonstrated that an increase in vasomotion can result in a rise in mean blood flow of 40-60%.[20,21] It seems therefore plausible that insulin-induced capillary recruitment may be mediated at least in part via effects on microvascular vasomotion. Indeed, several studies have demonstrated that insulin increases the intensity of vasomotion at the precapillary arteriolar level in the human[22] and in the rat muscle microvasculature,[14] as well as in the human cutaneous microvasculature.[23] Taken together, these data suggest that insulin, by altering arteriolar vasomotion and/or reducing prearteriolar tone, redirects blood flow from non-nutritive to nutritive capillary beds and thus increases insulin-mediated glucose uptake.

Obesity is associated with microvascular dysfunction

Microvascular function thus appears to be crucial in the regulation of muscle glucose metabolism and peripheral vascular resistance. Obesity, in turn, has been shown to be associated with several impairments in the microvasculature, which may be an important pathway through which obesity contributes to high blood pressure and insulin resistance. Obese individuals are characterized by muscle capillary rarefaction[24] and a disturbed balance of NO and ET-1 in the muscle microcirculation.[25,26] Also, obese individuals show reduced microvascular vasomotion under baseline conditions[27] and blunted responses in resistance arteries and arterioles to classic endothelium-dependent vasodilators.[26,28,29] In addition, several studies have demonstrated impaired insulin-induced vasodilation and insulin-mediated increases in microvascular perfusion in skin[12,28] and skeletal muscle[30] of obese individuals. Insulin's microvascular effects are impaired because, in obesity, insulin-mediated activation of the PI3-kinase pathway is impaired, while the activation of ERK1/2 and the production of ET-1 by insulin is normal. This results in an impaired vasodilator or even a net vasoconstrictor response of insulin on muscle resistance arteries and terminal

arterioles.[16] As a consequence, vascular resistance will increase, possibly contributing to elevations in blood pressure, as well as to extensive periods of poor delivery of insulin, glucose, and other nutrients to the muscle cells, contributing to impaired insulin-mediated glucose disposal in muscle.

The hypothesis that microvascular dysfunction may precede and predict the development of insulin resistance and hypertension is supported by the presence of microvascular dysfunction in normoglycemic, mildly overweight individuals with a genetic predisposition for type 2 diabetes,[31] and improvement of endothelial function by weight loss in obese women.[32] In addition, animal studies have demonstrated that diminished NO production, due to for example high-fat feeding, precedes the development of skeletal muscle insulin resistance,[33] diabetes, and hypertension.[34] In summary, these data support the notion that defects in microvascular function may contribute to impaired insulin-induced glucose uptake.

Microvascular function and steady-state versus dynamic (meal-induced) hyperinsulinemia

Most studies of the effect of insulin on microcirculatory function have been performed with the hyperinsulinemic euglycemic clamp technique, i.e. under steady-state hyperinsulinemia.[7,12-14,22,28,30,35] However, physiologically hyperinsulinemia is usually transient and dynamic, such as after a glucose load or after a meal, and is then accompanied by changes in circulating concentrations of glucose, amino acids, as well as gut and pancreatic peptides, which are not replicated by the clamp technique. If insulin's effects on microcirculatory function play a physiological role in regulating insulin-mediated glucose uptake, such effects should be demonstrable not only during steady-state hyperinsulinemia, but also after a glucose load and a meal. In addition, any such effects would be expected to be impaired in obese (insulin-resistant) individuals as compared to (insulin-sensitive) healthy controls.

In view of these considerations, we studied, in lean and obese individuals, the effects of an oral glucose load and a liquid mixed meal on cutaneous microvascular vasomotion.

Subjects and methods

Subjects

Eighteen lean and 13 obese Caucasian individuals participated (Table 5.1). Participants were healthy as judged by medical history, non-diabetic, as defined by an oral glucose tolerance test (OGTT), normotensive (<135/<85 mmHg), as determined by ambulatory 24h blood pressure monitoring (ABPM, SpaceLabs 90207, Redmond, WA, USA), and non-smokers. Participants did not use any medication except oral contraceptives (three lean and two obese women). All participants gave informed consent for participation in the study. The study was undertaken with the approval of the local ethics committee and performed in

accordance with the Declaration of Helsinki.

Study Design

All subjects were allocated to three interventions in random order, i.e. a glucose drink (75 g glucose in 250 ml water (similar to an OGTT)), a 495-kcal liquid mixed meal (volume: 330 ml, 60% carbohydrates, 25% proteins, 15% fat), or a control drink (300 ml tap water). Investigators were blinded with regard to the type of drink ingested. The interval between each of the interventions was one week.

All measurements were conducted in a temperature-controlled room (T=23.4±0.5C°) at 8.00 a.m., after a 10h fast, with the subjects in the supine position. Subjects were asked to refrain from drinking alcohol for a period of 24h before each study day and to perform no strenuous exercise for a period of 48h before each study day. Baseline measurements were obtained after allowing 30 min of rest and acclimatization after the insertion of one i.v. catheter (Venflon, Viggo, Gotenborg, Sweden).

Laser Doppler flowmetry

In order to perform vasomotion analyses, skin blood flow (in arbitrary perfusion units, PU) was measured with a laser Doppler probe (PF 457, Perimed, Stockholm, Sweden) at the dorsal side of the wrist of the dominant arm. Signals were recorded for 30 min, both at baseline and 30 min after intake of the glucose drink, the mixed meal drink, or water. Fast Fourier transform analysis was performed by means of Perisoft dedicated software (PSW version 2.50, Perimed, Sweden) in order to determine the contribution of the five frequency components to the variability of the laser Doppler signal (i.e. endothelial, 0.01-0.02 Hz; neurogenic, 0.02-0.06 Hz; myogenic, 0.06-0.15 Hz; respiratory, 0.15-0.40 Hz; and heart beat, 0.40-1.60 Hz).

Plasma insulin and glucose

Plasma insulin concentrations were measured by radioimmunoassay techniques (AutoDELFIA, PerkinElmer, Massachusetts, USA). Blood glucose concentrations were determined with a glucose analyzer YSI2300 (Yellow Springs Instrument, Yellow Springs, OH, USA). Insulin resistance was estimated by the calculation of the homeostasis model assessment-insulin resistance (HOMA2-IR) index.[36]

Statistical Analysis

Data are expressed as mean ± SD. A paired Student's *t* test was used to study effects of the different drinks, on glucose and insulin levels, within each group. The independent Student's *t* test was used to ascertain differences between the lean and obese groups.

The Wilcoxon signed-rank test for paired data was used to study differences in

vasomotion within each group between fasting and postprandial values. The Mann-Whitney test was used to compare effects of the interventions on vasomotion between the lean and obese group. A two-tailed P value of <0.05 was considered significant. All analyses were performed using the statistical software package SPSS version 15.0.

Results

Subject characteristics are shown in Table 5.1. By design, obese individuals had a significantly higher weight, body mass index and waist-to-hip ratio. The obese group had higher fasting insulin levels and were more insulin resistant in the fasting state (higher HOMA2-IR). In addition, 24h diastolic blood pressure was lower in obese individuals.

Table 5.1. Characteristics of lean and obese study groups

	Lean individuals	Obese individuals
Number (men/women)	18 (6/12)	13 (5/8)
Age (yr)	37.3 ± 12.9	35.9 ± 14.9
Weight (kg)	68.9 ± 10.3	100.3 ± 16.3 #
Body mass index (kg/m^2)	22.5 ± 1.7	34.0 ± 3.5 #
Waist-to-hip ratio	0.80 ± 0.08	0.90 ± 0.09 #
SBP daytime, 24h-ABPM (mmHg)	122 ± 7	119 ± 9
DBP daytime, 24h-ABPM (mmHg)	77 ± 5	73 ± 5 *
Fasting plasma glucose (mmol/l)	5.0 ± 0.6	5.1 ± 0.4
Fasting insulin (mU/l)	3.6 ± 1.0	10.4 ± 6.0 #
HOMA2-IR	0.46 ± 0.13	1.35 ± 0.75 #

Data are mean ± SD, or number. ABPM, ambulatory blood pressure monitoring. HOMA2-IR, homeostasis model assessment-insulin resistance. #P<0.01, *P<0.05 obese vs. lean individuals.

Effects of the glucose and mixed meal drink on plasma insulin and glucose levels

Glucose and insulin levels before and after ingestion of the different drinks in lean and obese subjects are shown in Figure 5.1.

After ingestion of the glucose drink, peak plasma glucose levels were higher compared with the mixed meal drink in both lean (P<0.05) and obese (P<0.001) individuals. Obese individuals, compared to lean individuals, had higher glucose levels after both glucose ingestion and the mixed meal (P<0.001 and P<0.05, respectively) (Figure 5.1A). In lean individuals, but not in the obese, plasma insulin levels rose to higher levels after ingestion of the mixed meal drink as compared to the glucose drink (P<0.01). In addition, peak insulin levels after both the glucose and the mixed meal drink reached higher levels in obese compared to lean individuals (P<0.05, P=0.07, respectively) (Figure 5.1B). Water intake did not alter plasma glucose or insulin levels in both groups.

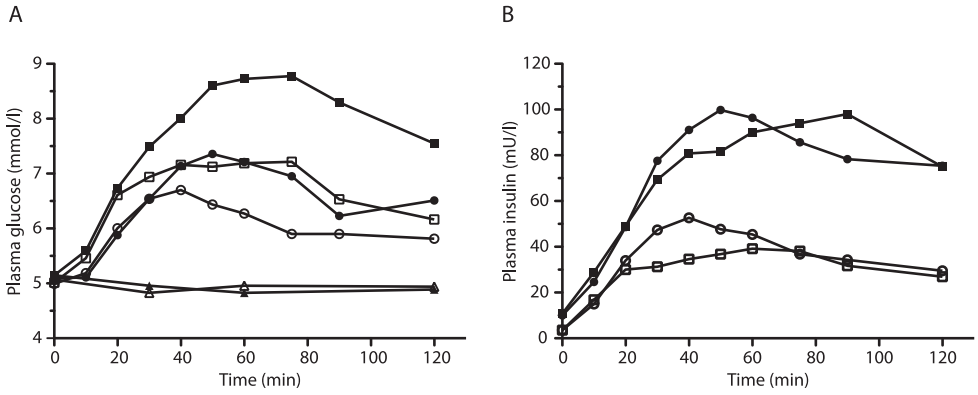

Figure 5.1. A: Time course of mean plasma glucose levels in lean (open symbols) and obese (closed symbols) individuals after the glucose drink (□,■), the mixed-meal drink (○,●), and water (△,▲). B: Time course of mean plasma insulin levels in lean (open symbols) and obese (closed symbols) individuals after the glucose (□,■) and the mixed-meal drink (○,●).

Effects of the glucose and mixed meal drink on microvascular vasomotion

At baseline, microvascular vasomotion was not different between lean and obese individuals (baseline energy density (mean of three study days) for lean vs. obese individuals for each of the five frequency bands and the total spectrum: 0.62±0.35 vs. 0.49±0.15 (P=0.49); 0.45±0.18 vs. 0.41±0.13 (P=0.60); 0.23±0.11 vs. 0.23±0.21 (P=0.41); 0.09±0.04 vs. 0.07±0.04 (P=0.16); 0.07±0.03 vs. 0.06±0.03 (P=0.23); and 1.46±0.67 vs. 1.25±0.45 Hz (P=0.44), respectively).

Microvascular flow (PU) did not change during any of the experiments (data not shown). Water intake did not alter the energy density of any of the five frequency components in either group (data not shown).

In lean individuals, the glucose drink increased the energy density of the frequencies between 0.01 and 0.02 Hz (Figure 5.2A). Intake of the liquid mixed meal increased the energy density of all five frequency bands, and the total energy density of the entire spectrum (Figure 5.2B). In obese individuals, neither the glucose (Figure 5.2C) nor the mixed meal drink (Figure 5.2D) had any effect on the energy density of the five frequency bands or the total energy density of the entire spectrum.

After the mixed meal, but not after glucose, obese individuals had lower increases in the energy density of the frequency bands between 0.01-0.02, 0.02-0.06, and 0.15-0.40 Hz, as well as in the total energy density of the entire spectrum as compared to lean individuals (-0.03±0.27 vs. +0.20±0.21, P<0.01; +0.05±0.19 vs. +0.17±0.21, P<0.05; 0.00±0.04 vs. +0.07±0.09, P<0.05; and 0.00±0.60 vs. +0.57±0.61, P<0.01, respectively).

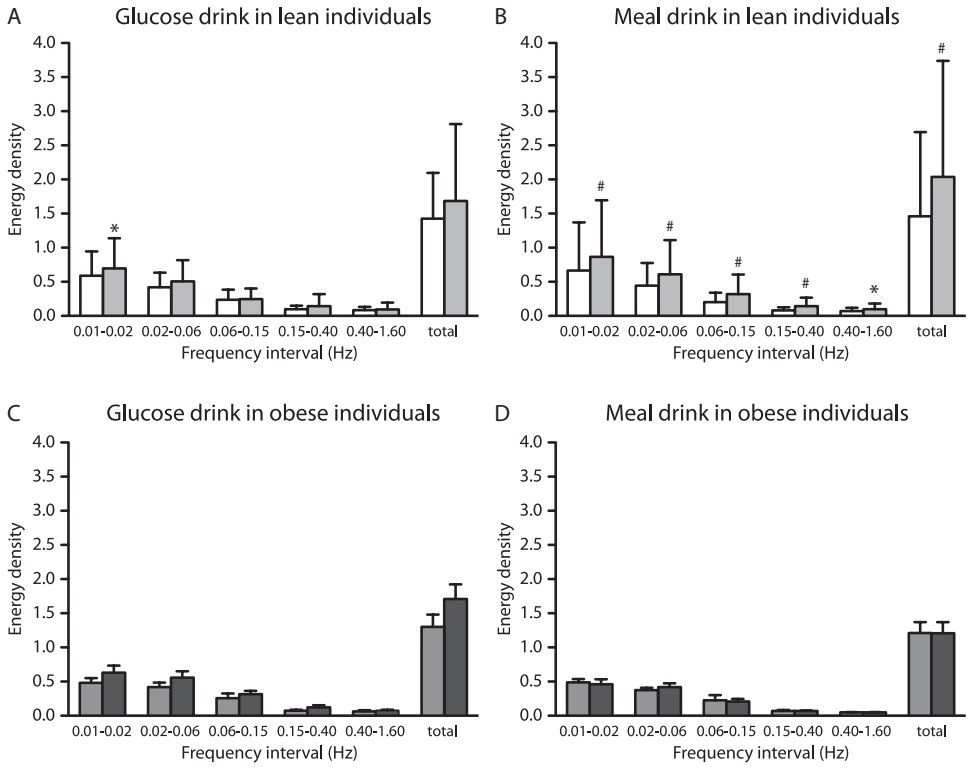

Figure 5.2. Microvascular vasomotion before (□) and after (▒) the glucose drink (A) and the mixed meal drink (B) in lean subjects. Microvascular vasomotion before (▒) and after (■) the glucose drink (C) and the mixed meal drink (D) in obese subjects. Bars present mean ± SD. #P<0.01, *P<0.05 vs. before intake.

Discussion

This study, in lean and (insulin-resistant) obese individuals, had three main findings. First, in the basal, preprandial state, there were no significant differences in microvascular vasomotion between lean and obese individuals. Second, ingestion of a glucose drink and a liquid mixed meal drink induced an increase in microvascular vasomotion in lean individuals. Third, the increase in microvascular vasomotion after a meal was impaired in obesity. In addition, the lack of meal-induced stimulation of microvascular vasomotion in obese individuals was associated with elevated plasma glucose levels (e.g. attenuated insulin-stimulated glucose disposal) after meal feeding in these subjects. These findings are consistent with a role for microvascular dysfunction, specifically, impaired vasomotion, in the development of obesity-related insulin resistance.

Our finding that, in the basal state, the energy density within each of the five frequency bands and that of the total spectrum was, although numerically smaller in obese than in lean individuals, not significantly different between these groups contrasts with findings of de Jongh et al.,[27] who demonstrated significantly lower energy densities within the frequency intervals 0.01-0.02 Hz and 0.02-0.06 Hz and reduced energy density of the total frequency spectrum in obese compared to lean women. The explanation for this discrepancy is not entirely clear, but may be related to the fact that the individuals in the current study were less obese than in the previous study,[27] and to the fact that we studied a smaller group of obese individuals, i.e. had less power to detect small differences.

There is convincing evidence that insulin-induced changes in skeletal muscle microvascular blood flow distribution play an important role in regulating muscle glucose disposal.[5] Herein, we propose a significant role for insulin-induced increases in microvascular vasomotion. Indeed, several studies have demonstrated that insulin increases the intensity of vasomotion at the precapillary arteriolar level in the human[22] and in the rat muscle microvasculature,[14] as well as in the human cutaneous microvasculature.[23] However, until now, these effects of insulin on microvascular vasomotion have only been measured during an insulin clamp[14,22] or local application of insulin to the surface of the skin[23] and not postprandially. In the present study, we are the first to demonstrate that ingestion of glucose or a mixed meal indeed stimulates microvascular vasomotion in healthy lean individuals. Likely, this effect may be secondary to meal-induced hyperinsulinemia, as indicated by the larger increase in both insulin levels and microvascular vasomotion after the mixed meal drink compared to the glucose drink in lean individuals. However, given the complex neuroendocrine responses to feeding, and the effects of different meal components (i.e. fat, carbohydrates, proteins) thereupon,[37] we cannot exclude other factors contributing to the postprandial changes in microvascular vasomotion.

Another key finding in this study was that the increase in microvascular vasomotion with meal ingestion was impaired in insulin-resistant obese individuals. In addition, this lack of meal-induced stimulation of microvascular vasomotion paralleled blunted insulin-stimulated glucose disposal (i.e. elevated plasma glucose levels) after meal feeding in obese individuals. This link between meal-induced microvascular vasomotion and insulin-stimulated glucose uptake is consistent with a role for insulin-stimulated microvascular vasomotion in insulin-mediated glucose uptake in daily life, and consequently with a role for microvascular dysfunction in the development of insulin resistance in obesity.

A possible limitation of the present study is that although muscle is the main peripheral site of insulin-induced microvascular function and insulin-mediated glucose uptake, we studied skin and not muscle microvascular vasomotion. However, there is substantial evidence that skin cutaneous microcirculation is representative of microcirculation in general (e.g. skeletal muscle). This is underscored by several studies demonstrating that

cutaneous microvascular function mirrors generalized systemic microvascular function.[38] With regard to insulin infusion, several studies have demonstrated comparable metabolic[39] and vascular effects[12,13] of insulin in muscle and skin. In addition, skin microvascular vasodilator capacity is associated with both insulin's vascular and metabolic[40] actions in skeletal muscle. The latter is clearly demonstrated in a study in which acutely increasing or decreasing skin nutritive flow has been shown to result in parallel changes in skeletal muscle insulin-mediated glucose uptake.[12]

Possible pathophysiological mechanisms for obesity-associated microvascular dysfunction

The pathophysiological mechanisms behind the relationship between obesity and microvascular dysfunction are probably multifactorial. Adipose tissue and in particular visceral adipose tissue cells secrete a variety of hormones and cytokines, such as FFAs, tumor necrosis factor-α (TNF)-α, interleukin-6, adiponectin, leptin, and angiotensinogen.[1] Some of these adipokines have been shown to affect microvascular function. For example, elevation of FFAs has been demonstrated to impair endothelium-dependent vasodilation,[41,42] at least in part through decreased NO production.[41] Also, both FFA and TNFα directly inhibit insulin-mediated capillary recruitment and insulin-mediated glucose disposal in rat skeletal muscle.[10,11,43] In addition, in lean individuals, FFA elevation impairs insulin-mediated stimulation of microvascular flow[12,13,41] and these impairments correlate closely with inhibition of insulin-mediated muscle glucose uptake.[12,13] Conversely, lowering of FFA in obese humans has an opposite effect.[12] Also, circulating TNFα levels are associated with impaired skin capillary recruitment,[44] and weight loss resulted in a significant amelioration of endothelial function that closely correlated with a reduction in circulating TNFα.[32] Thus, FFAs and TNFα have been shown to contribute to microvascular insulin resistance. This effect may be due to inhibiting effects on the insulin-signaling cascade. Both FFA and TNFα elevation have been demonstrated to blunt insulin-induced PI3-kinase activation directly, or via the production of reactive oxygen species (ROS).[45-49] Also, both FFA and TNFα upregulate the expression of ET-1 in human endothelial cells,[35,50,51] while TNFα downregulates the expression of eNOS.[52] Thus, circulating FFAs and TNFα are likely candidates to link adipose tissue with defects in microvascular function, at least in part by influencing insulin signaling and thereby the vascular effects of insulin. In addition, locally produced adipokines from perivascular fat depots may directly inhibit vasodilatory pathways distal in the arteriole and thereby cause loss of blood flow in the nutritive capillary network supplied by this arteriole.[53,54] Nevertheless, whether postprandial changes in FFA and TNFα concentrations are involved in the impairment of meal-induced vasomotion in obesity cannot be derived from the current data, and further studies in this area are needed.

Another potential mechanism between adipose tissue and the microvasculature is the renin-angiotensin system (RAS). Obese individuals are characterized by increased

activity of the RAS.[55] Multiple mechanisms may contribute to RAS-induced vascular insulin resistance. Angiotensin II (AngII) has been demonstrated to blunt insulin-induced PI3-kinase activation[56] directly, or via stimulated ROS generation.[57] In addition, AngII has been shown to activate the ERK 1/2 pathway and consequently stimulates ET-1 production. Support for a role of AngII in insulin-mediated microvascular dysfunction is provided by a study in the Zucker obese diabetic rat, in which chronic angiotensin converting enzyme inhibition improved insulin-mediated capillary recruitment and insulin-mediated glucose disposal.[58] Therefore, we hypothesize that AngII may play an important role in compromising microvascular function and thus provide another potential link between obesity and obesity-related microvascular dysfunction. Whether systemic or local RAS activity can be regulated by nutritional factors is not known. Nevertheless, several studies have demonstrated that acute hyperinsulinemia (i.e. an insulin clamp) increases plasma renin and AngII levels and upregulates angiotensinogen mRNA expression in adipose tissue.[59] This increase in systemic RAS activity with insulin is likely mediated via sympathetic activation,[60] which has been shown to be more pronounced in obese compared to lean individuals.[60]

In summary, we are the first to report that ingestion of a meal increases microvascular vasomotion in healthy lean individuals. This increased vasomotion is likely to play an important role in skeletal muscle blood flow distribution and, therefore, the regulation of insulin and glucose delivery to skeletal muscle. In addition, we demonstrated that the increase in microvascular vasomotion with meal ingestion was impaired in obese individuals. This lack of meal-induced stimulation of microvascular vasomotion paralleled blunted insulin-stimulated glucose disposal after meal feeding in these subjects, which again is consistent with a role for insulin-stimulated microvascular vasomotion in insulin-mediated glucose uptake in the postprandial state. This paper has further reviewed the evidence for microvascular dysfunction as a key element in the development of obesity-related hypertension and insulin resistance, and consequently in the development of cardiovascular disease and type 2 diabetes. We propose that, in obesity, microvascular dysfunctions may be the result of alterations in endocrine and vasocrine[1,53] signaling, in which adipokines and the RAS play a prominent role. Additional studies are required to fully understand the precise mechanisms that link obesity to insulin-mediated microvascular dysfunction. Such studies are an important step towards development of strategies in the prevention and treatment of obesity-associated hypertension and insulin resistance.

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6

Obesity is associated with impaired endothelial function in the postprandial state

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Abstract

Adequate microvascular perfusion is essential for the regulation of tissue metabolism. Therefore, defects in microvascular function may play a role in obesity-associated insulin resistance. Steady-state hyperinsulinemia during a euglycemic hyperinsulinemic clamp stimulates endothelium-dependent vasodilation and capillary recruitment, which contribute to increased glucose uptake. These phenomena have been shown to be blunted in obesity. If insulin's effects on microcirculatory function indeed play a physiological role in regulating insulin-mediated glucose uptake, such effects should be demonstrable not only during steady-state hyperinsulinemia, but also after meal ingestion. We investigated whether similar responses occur after ingestion of a glucose load or a mixed meal. We examined the effects of a glucose drink, a mixed meal drink, or a control drink (water) on skin capillary density (i.e. baseline capillary density, hyperemic capillary recruitment, and density during venous congestion, using capillaroscopy) and skin endothelium-(in)dependent vasodilation (using laser-Doppler flowmetry with iontophoresis of acetylcholine and sodium nitroprusside) in 20 lean and 19 obese individuals. In lean individuals, neither the glucose nor the mixed meal drink induced a significant effect on capillary density or endothelium-(in)dependent vasodilation. Possibly this is related to the modest plasma insulin levels as compared to the insulin clamp. In obese individuals, the mixed meal drink, compared to the control drink, decreased baseline skin perfusion ($P<0.05$) and acetylcholine-mediated vasodilation ($P<0.05$), while no effect of the drinks on capillary density was found. Compared to lean individuals, obese individuals had impaired acetylcholine-mediated vasodilation after meal ingestion ($P=0.02$). The latter findings are consistent with impaired postprandial microvascular function in obesity.

Introduction

Obesity is associated with insulin-resistance. This association is not completely elucidated, but might be related to obesity-associated microvascular dysfunction and subsequent impaired delivery of insulin and glucose to muscle cells.[1]

There is increasing evidence, that, in addition to its essential metabolic actions, insulin has important vascular actions that involve vasodilation of precapillary arterioles, leading to an increased number of capillaries perfused, and enhanced access of glucose and insulin to muscle cells.[2-4] It has been shown that this microvascular action of insulin accounts for ~40% of insulin-stimulated muscle glucose uptake.[5-7] Therefore, failure of this microvascular action may be one factor that contributes to postprandial hyperinsulinemia, associated with obesity.

Using capillary microscopy in healthy lean individuals, we have found that systemic hyperinsulinemia, as induced by the hyperinsulinemic euglycemic clamp technique, indeed increases the number of perfused skin capillaries.[8-11] In addition, we have demonstrated that this response is blunted in obesity.[8] However, although the clamp technique provides an excellent assessment of responsiveness to physiological hyperinsulinemia, it does not mimic the physiological responses that typically occur after the ingestion of a meal. Specifically, postprandial hyperinsulinemia is usually transient and dynamic and accompanied by, among others, changes in circulating concentrations of glucose, amino acids, and gut and pancreatic peptides, and changes in parasympathetic and sympathetic tone. If insulin's effects on microcirculatory function play a physiological role in regulating insulin-mediated glucose uptake, such effects should be demonstrable not only during steady-state hyperinsulinemia, but also after a glucose load and a meal. In addition, any such effects would be expected to be impaired in obese (insulin-resistant) individuals as compared to (insulin-sensitive) healthy controls.

Therefore, the present study, in lean and obese individuals, was designed to examine whether microvascular responses similar to those obtained during an insulin clamp can be obtained by ingestion of a glucose load or a mixed meal.

Subjects and methods

Subjects

Twenty lean (body mass index<25 kg/m^2) and 19 obese (body mass index>30 kg/m^2) individuals participated in this study. All subjects were Caucasian, non-smokers, non-diabetic, as defined by an oral glucose tolerance test (OGTT), normotensive (<135/<85 mmHg), as determined by ambulatory 24h blood pressure monitoring, and had no signs or symptoms of cardiovascular or other concomitant disease. None of the participants used any medication during the studies except for oral contraceptives (three lean and two obese women). All participants gave informed consent for the study. The study was undertaken

with the approval of the local ethics committee and performed in accordance with the Declaration of Helsinki.

Study design

All subjects were allocated to three interventions in random order, i.e. a glucose drink (75g glucose in 250 ml water (similar to an OGTT)), a standard 495-kcal liquid mixed meal (60% carbohydrates, 25% proteins, 15% fat; 240 ml Nutridrink® and 90 ml Nutridrink Juice style®, Nutricia, Zoetermeer, The Netherlands), or a control drink (300 ml tap water). Investigators were blinded with regard to the type of drink ingested. The interval between each of the interventions was one week. Dietary sodium intake was assessed by the collection of two 24h urine samples, separated by one week.

All measurements were conducted in a temperature-controlled room (T=23.4±0.5 °C) at 8.00 a.m., after a 10h fast, with the subjects in the supine position. Subjects were asked to refrain from drinking alcohol for a period of 24h before each study day and to perform no strenuous exercise for a period of 48h before each study day. Baseline measurements of endothelium-(in)dependent vasodilation (laser-Doppler flowmetry) and skin capillary density (capillary microscopy) were obtained after allowing 30 min of rest and acclimatization after the insertion of one i.v. catheter (Venflon, Viggo, Gotenborg, Sweden). At 30 min after ingestion of the glucose drink, the mixed meal drink, and the control (water) drink a second measurement of endothelium-(in)dependent vasodilation and, at 75 min, a second measurement of capillary density were performed (Figure 6.1). A period of 30 min after drink ingestion was chosen because previous studies demonstrated that plasma insulin concentrations reach peak values within 30-60 min after both glucose load or liquid mixed meal ingestion, after which the levels slowly decrease.[12-14] Skin temperature was monitored during the tests.

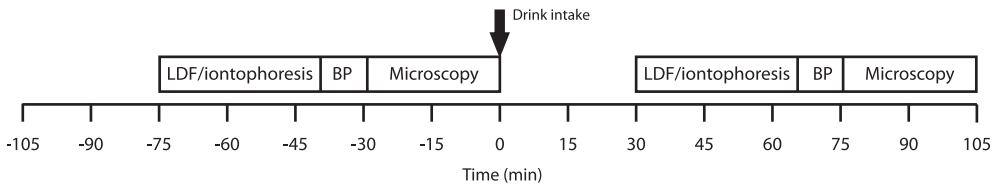

Figure 6.1. Design of the study. LDF/iontophoresis indicates endothelium-(in)dependent vasodilation during iontophoresis of acetylcholine and sodium nitroprusside in the skin of the finger; BP, blood pressure measurement; Microscopy, capillary microscopy of the skin of the finger; ↓, ingestion of the glucose drink, the mixed meal drink and the control drink.

Capillary microscopy

Nailfold capillary studies were performed at baseline (*t* = -30-0 min) and after intake of

the glucose drink, the mixed meal drink, or the control drink (t = 75-105 min). Nailfold capillaries in the dorsal skin of the fourth finger were visualized by a capillary microscope (Leitz-Orthoplan) in combination with a CCD-camera. To visualize the capillaries, a 4.0 objective (Leitz N.A. 0.14) was used with a total system magnification of 160x. Capillaries were visualized ~4.5 mm proximal to the terminal row of capillaries in the middle of the nailfold. Subsequently, a characteristic capillary was kept on the same spot of the visual field to ensure that capillary density was measured in the exact same visual field during the entire experiment. A visual field of 1 mm^2 was recorded before and after 4 min of arterial occlusion with a digital cuff. Capillaries at baseline and directly after release of the cuff were counted for, respectively, 30 and 45 s. Baseline capillary density was defined as the number of continuously erythrocyte-perfused capillaries per square millimeter of nailfold skin. Other capillaries can be seen to be intermittently perfused, and these may represent an important functional reserve. Postocclusive reactive hyperemia (PRH) after 4 min of arterial occlusion was used to assess this functional reserve capacity. Percentage capillary recruitment was calculated by dividing the increase in capillary density during postocclusive reactive hyperemia by the baseline capillary density. In addition, we applied venous congestion, with the digital cuff inflated to 60 mmHg for 60 s, to expose a maximal number of capillaries.[15] All procedures were performed on two separate visual fields and the mean of both measurements was used for analyses. A single experienced investigator performed all measurements in order to avoid inter-observer variability in recordings. Capillaries were counted by the investigator and an observer who were both blinded to the characteristics of the subjects and to the experimental status of the recordings. Both observers counted approximately half of the individuals in the lean group and half of the individuals in the obese group. The inter-observer variability was 7.2%. The day-to-day coefficients of variation of baseline capillary density, peak capillary recruitment, and peak density during venous congestion varied between 3.4 and 7.4%, as determined on 2 separate days.

Laser Doppler flowmetry

Endothelium-dependent and endothelium-independent vasodilation were evaluated with Laser Doppler flowmetry (Periflux 5000, Perimed, Stockholm, Sweden) in combination with iontophoresis of acetylcholine and sodium nitroprusside, respectively.[15] Measurements were performed at baseline (t = -75 to -35 min) and after intake of the glucose drink, the mixed meal drink, or the control drink (t = 30 to 70 min). Acetylcholine (1% Miochol, Novartis Pharma GmbH, Nürnberg, Germany) was delivered on the middle phalanx of the second finger of the left hand using an anodal current (seven doses of 0.1 milliamps for 20 s with a 60-s interval). Sodium nitroprusside (0.01% Nipride, Radboud Hospital, Nijmegen, The Netherlands) was delivered on the middle phalanx of the fourth finger of the left

hand using a cathodal current (nine doses of 0.2 milliamps for 20 s with a 90-s interval). The responses were calculated as absolute and percentage increases from baseline to plateau phase. The day-to-day coefficients of variation of acetylcholine- and sodium nitroprusside-dependent vasodilation were 22.4% and 23.5%, respectively, as determined in 15 individuals on two separate days. Skin temperature was above 30°C and monitored continuously during all measurements.

Blood pressure

Ambulatory monitoring (SpaceLabs 90207, Redmond, WA, USA) was used to obtain 24h recordings of blood pressure and heart rate. During study days, blood pressure and heart rate were determined at the times specified in Figure 1 by means of an automatic device (Datascope Accutorr Plus™, Paramus, NJ, USA).

Plasma insulin and glucose

Plasma insulin concentrations were measured by radioimmunoassay techniques (AutoDELFIA, PerkinElmer, Massachusetts, USA). Blood glucose concentrations were determined with a glucose analyzer YSI2300 (Yellow Springs Instrument, Yellow Springs, OH, USA). Insulin resistance was estimated by the calculation of the homeostasis model assessment-insulin resistance (HOMA2-IR) index.[16]

Statistical analysis

For baseline capillary density we calculated beforehand a sample size of n=20 to be sufficient to find a 5% change in capillary density with a two-sided α-level <0.05 and a power of 80%. In addition, it was calculated that this number would suffice to find a 35% change in endothelium-(in)dependent vasodilation with a two-sided α-level <0.05 and a power of 80%.

Data are expressed as mean ± SD or median (interquartile range) as appropriate. The paired Student's *t* test and the Wilcoxon signed-rank test for paired data were used to study effects of the different drinks within lean and obese individuals. The independent Student's *t* test and the Mann-Whitney test were used to compare effects of the drinks between lean and obese individuals. A two-tailed P value of <0.05 was considered significant. All analyses were performed using the statistical software package SPSS version 15.0.

Results

By design, lean individuals had a significantly lower body weight, body mass index, and waist-to-hip ratio. The obese group had higher fasting plasma insulin levels and lower insulin sensitivity in the fasting state (higher HOMA2-IR; Table 6.1).

Table 6.1. Characteristics of lean and obese study groups

	Lean individuals	Obese individuals
Number (men/women)	20 (7/13)	19 (5/14)
Age (years)	32.0 (25.3-44.0)	35.0 (23.0-54.0)
Weight (kg)	69.2 (62.5-76.6)	94.0 (85.5-106.0) *
Body mass index (kg/m^2)	22.9 (21.0-23.8)	33.5 (31.0-35.2) *
Waist-to-hip ratio	0.80 (0.74-0.86)	0.89 (0.85-0.92) #
24h Systolic blood pressure (mmHg)	116 (111-121)	118 (113-122)
24h Diastolic blood pressure (mmHg)	73 (68-74)	71 (68-75)
24h Heart rate (bpm)	71 (61-78)	73 (65-80)
Fasting plasma glucose (mmol/l)	4.9 (4.4-5.2)	5.0 (4.5-5.3)
Fasting insulin (pmol/l)	3.1 (3.0-3.8)	8.2 (6.0-11.2) *
HOMA2-IR	0.40 (0.40-0.50)	1.10 (0.80-1.50) *
Urinary sodium excretion (mmol/24h)	125.0 (104.6-158.5)	135.5 (113.5-176.5)

Data are medians (25–75%), or number, HOMA2-IR; homeostasis model assessment-insulin resistance. *P<0.001, #P<0.01 vs. lean individuals.

Effects of the glucose and mixed meal drinks on plasma insulin and glucose levels

Following ingestion of the glucose drink, the area under the glucose curve was higher as compared to the mixed meal drink in both lean (P<0.01) and obese individuals (P<0.01). Compared to lean individuals, obese individuals had a higher postprandial area under the glucose and insulin curves, after both the glucose (P<0.05 and P<0.001) and the mixed meal drink (P<0.05 and P<0.001). In lean individuals, but not in the obese group, peak plasma insulin levels rose to higher levels after ingestion of the mixed meal drink as compared to the glucose drink (P<0.01). Ingestion of the control drink did not alter plasma glucose levels in either group (Figure 6.2).

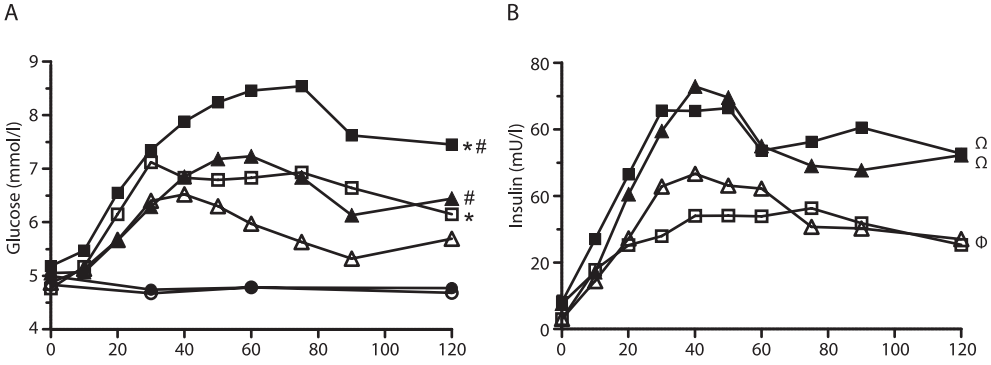

Figure 6.2. Time course of plasma glucose levels (A) and plasma insulin levels (B) in lean (open symbols) and obese (closed symbols) individuals after the glucose drink (□, ■), the mixed meal drink (Δ, ▲), and the control drink (○, ●). Data are expressed as medians. Significance for comparison of glucose and insulin areas under the curve 0-120 min: *P<0.01 vs. mixed meal drink within group. Ω P<0.001, # P<0.05 vs. lean individuals. Φ P<0.01 vs. peak plasma insulin levels during mixed meal drink in lean individuals.

Effects of the glucose and mixed meal drinks on capillary density

Due to technical difficulties with the videomicroscopy we were unable to evaluate the effects of the different drinks on capillary density in one lean individual.

Within the lean and the obese groups, baseline capillary density, hyperemic capillary recruitment, and density during venous congestion did not differ significantly before the glucose drink, the mixed meal drink, and the control drink (Table 6.2). At baseline, these variables also did not differ significantly between lean and obese individuals.

Both in lean and in obese individuals, neither the glucose, the mixed meal, nor the control drink induced a significant effect on baseline capillary density, hyperemic capillary recruitment or density during venous congestion (Table 6.2). Skin temperature was not significantly different between lean and obese individuals or during the experiments (data not shown).

Table 6.2. Changes in nailfold capillary densities after intake of the glucose drink, the mixed meal drink, and the control drink in lean and obese individuals

	Pre glucose	ΔGlucose	Pre meal	ΔMeal	Pre control	ΔControl
Lean individuals						
Baseline density (n/mm^2)	58.4 ± 12.6	-1.6 ± 5.1	57.2 ± 11.7	-1.0 ± 4.5	54.2 ± 9.4	-0.7 ± 3.1
Recruitment PRH (%)	14.7 ± 8.1	+4.8 ± 12.5	15.9 ± 10.5	+2.5 ± 10.9	16.6 ± 8.1	+2.5 ± 11.9
Venous occlusion (n/mm^2)	73.0 ± 12.4	+0.5 ± 4.6	73.5 ± 11.3	+0.7 ± 4.3	70.6 ± 7.9	+0.6 ± 3.3
Obese individuals						
Baseline density (n/mm^2)	61.1 ± 14.1	-1.0 ± 2.3	59.8 ± 14.4	+0.3 ± 4.1	62.0 ± 14.0	-0.7 ± 3.5
Recruitment PRH (%)	16.6 ± 8.1	+1.7 ± 4.6	19.1 ± 9.8	-1.7 ± 8.3	13.9 ± 7.2	+3.5 ± 10.1
Venous occlusion (n/mm^2)	79.1 ± 14.4	-1.2 ± 4.4	77.9 ± 13.9	-0.8 ± 3.1	78.6 ± 15.6	+0.2 ± 2.6

Data are mean ± SD. n/mm^2; number of perfused capillaries per square millimeter. PRH, peak reactive hyperemia. Δs represent changes in nailfold capillary densities due to the glucose drink, the mixed meal drink, and the control drink.

Effects of the glucose and mixed meal drinks on endothelium-(in)dependent vasodilation

Within the lean and obese groups, baseline skin perfusion before iontophoresis of either acetylcholine or sodium nitroprusside as well as the responses to iontophoresis of acetylcholine and sodium nitroprusside were comparable before intake of the glucose drink, the mixed meal drink, or the control drink. At baseline, these variables also did not differ between lean and obese individuals (Table 6.3).

In both lean and obese individuals, baseline skin perfusion decreased during all three study days in a similar fashion.

Compared to the control drink, ingestion of the mixed meal significantly decreased baseline skin perfusion and acetylcholine-mediated vasodilation in obese, but not in lean individuals. The meal-induced decrease in percentage acetylcholine-mediated vasodilation in obese individuals was significantly different from the meal-induced increase in percentage acetylcholine-mediated vasodilation in lean individuals (median (interquartile

range) -18.5% (-56.8 to +13.3) vs. +56.5% (-0.8 to +242.9), P=0.02). Compared to the control drink, ingestion of the glucose drink did not induce a significant effect on acetylcholine-mediated vasodilation in either lean or obese individuals. Compared to the control drink, ingestion of the glucose drink and the mixed meal drink did not significantly alter sodium nitroprusside-mediated vasodilation in either lean or obese individuals.

Table 6.3. Endothelium-(in)dependent vasodilation in the skin of lean and obese individuals before and after intake of the glucose drink, the mixed meal drink, and the control drink

	Pre glucose	ΔGlucose	Pre meal	ΔMeal	Pre control	ΔControl
Lean individuals						
Ach-mediated vasodilation						
Baseline skin perfusion (PU)	37.0 ± 17.9	-16.9 ± 17.3	39.0 ± 17.9	-17.1 ± 18.0	36.9 ± 16.8	-9.7 ± 18.3
Plateau (PU)	87.2 ± 55.7	-22.6 ± 52.8	89.1 ± 59.2	-19.3 ± 62.7	92.3 ± 55.2	-9.4 ± 68.0
SNP-mediated vasodilation						
Baseline skin perfusion (PU)	28.5 ± 16.5	+0.77 ± 21.1	40.2 ± 22.9	-7.0 ± 30.8	35.8 ± 18.5	-8.2 ± 14.2
Plateau (PU)	122.0 ± 77.7	-17.4 ± 55.3	163.8 ± 78.4	-48.1 ± 66.9	147.9 ± 83.1	-38.5 ± 52.9 #
Obese individuals						
Ach-mediated vasodilation						
Baseline skin perfusion (PU)	38.0 ± 16.7	-13.9 ± 13.7	40.2 ± 18.6	-16.8 ±17.0 *	35.1 ± 15.6	-9.2 ± 9.2
Plateau (PU)	77.7 ± 39.6	-21.0 ± 40.2	94.9 ± 70.2	-45.1 ± 59.5 *	71.1 ± 46.6	-9.5 ± 39.0
SNP-mediated vasodilation						
Baseline skin perfusion (PU)	34.8 ± 22.7	d-11.3 ± 26.0	36.8 ± 16.0	-10.1 ± 20.3	38.7 ± 21.7	-16.7 ± 26.2
Plateau (PU)	125.1 ± 70.8	-33.9 ± 55.3	117.9 ± 75.6	-12.9 ± 48.0	117.7 ± 71.2	+1.8 ± 60.2

Data are mean ± SD. Ach, acetylcholine; SNP, sodium nitroprusside; PU, arbitrary perfusion units. Δs represent changes in endothelium-(in)dependent vasodilation due to the glucose drink, the mixed meal drink, and the control drink.*P<0.05 vs. change due to control intake within subject group. #P<0.05 vs. change due to control in obese individuals.

Effects of the glucose and mixed meal drink on blood pressure and heart rate

Within the lean and the obese groups, baseline blood pressure and heart rate were comparable during the three visits. Baseline hemodynamic variables also did not differ significantly between lean and obese individuals.

Compared to the control drink, ingestion of the glucose drink and the mixed meal drink did not significantly alter blood pressure, either in lean or in obese individuals. Compared to the control drink, ingestion of the glucose drink and the mixed meal drink significantly increased heart rate to a similar extent in both groups (data not shown).

Discussion

This study, in lean and obese (more insulin-resistant) individuals, has two main findings. First, unlike findings with the hyperinsulinemic clamp technique, ingestion of a glucose load or a mixed meal did not change perfused capillary density (i.e. baseline capillary density, hyperemic capillary recruitment, or capillary density during venous congestion)

or endothelium-(in)dependent vasodilation in lean individuals. Second, ingestion of the mixed meal blunted endothelium-dependent vasodilatory reactivity in obese individuals, while no effect of the glucose load or the mixed meal on perfused capillary density was found.

It has become increasingly evident that insulin itself is an important vasoregulatory hormone which can enhance muscle microvascular perfusion as an integral part of its action to increase skeletal muscle glucose disposal.[2-4] Therefore, defects in, specifically, muscle microvascular function or insulin's effects thereon may contribute to impaired insulin-induced glucose uptake.[1,5,6,10,17] Muscle microvascular responses to physiological hyperinsulinemia have, however, been difficult to assess in humans due to the lack of non-invasive techniques. Nevertheless, the cutaneous circulation has emerged as a valuable model of the microcirculation and may be considered as representative of the microcirculation in skeletal muscle.[18] Therefore, over the past 20 years, we and others have used intravital capillary microscopy and iontophoresis of acetylcholine in the skin to study skin microvascular function in response to insulin.[8-11] Using these techniques, we have repeatedly shown that systemic hyperinsulinemia, induced by the hyperinsulinemic clamp technique, increases the number of perfused nutritive capillaries and augments endothelium-dependent vasodilation in skin.[8-11] Importantly, these hyperinsulinemia-induced capillary responses in skin were strongly correlated with skeletal muscle glucose metabolism.[8,10] However, since the clamp does not completely mimic postprandial hyperinsulinemia, the objective of the present study was to examine whether similar microvascular responses could be obtained in a more physiological setting, i.e. after a meal or an oral glucose load. Within this setting we were not able to detect significant changes in nutritive microvascular perfusion in the skin of lean healthy individuals. This is likely to be related to the lower rise in plasma insulin (~35mU/l) and the more dynamic nature of the insulin response after the meal and glucose load as compared to the response after intravenous insulin infusion in previous studies (i.e. ~60mU/l).[8-11] Alternatively, this lack of response might also be related to the more complex neuroendocrine output that derives from meal ingestion. Unlike simple insulin infusion, feeding provokes a complex, systemic response involving the changes in circulating concentrations of nutrients such as glucose and amino acids, gut and pancreatic peptides, and autonomic nervous system tone. These responses may modify insulin's vasodilatory effects, as a result of which no net increase in microvascular perfusion is found. However, in a previous study, using another technique (i.e. Fourier analysis of skin microcirculatory blood flow), we did find an increase in skin microvascular vasomotion in lean individuals after ingestion of both a glucose load and a mixed meal.[19] In addition, recent studies using contrast-enhanced ultrasound demonstrated an increased microvascular blood volume within the skeletal muscle of lean individuals after ingestion of a mixed meal.[12,20,21] Notably,

in two of these studies increases in microvascular blood volume were reported 120 min after meal ingestion and not earlier.[20,21] Thus, these studies, using other techniques for defining microvascular perfusion and/or distribution, did demonstrate an increase in microvascular perfusion with meal ingestion. Taken together, these[12,19-21] and the current data suggest that the specific changes in nutritive microvascular perfusion in lean individuals during dynamic postprandial hyperinsulinemia are not detected using skin capillary density and skin endothelium-(in)dependent reactivity, possibly because these techniques are not sufficiently sensitive to detect such changes or because of the timing of the microvascular measurements performed.

In the present study, endothelium-dependent vasodilation during mixed meal ingestion was blunted in obese compared to lean individuals. This is in agreement with previous studies in both skin and skeletal muscle, in which obese, compared to lean individuals, demonstrated blunted endothelium-dependent reactivity and capillary recruitment after insulin infusion[8,10,22,23] or mixed meal ingestion.[20] In addition, a potentially important finding in the present study was that meal ingestion in itself diminished baseline skin perfusion and endothelial function in obese individuals, an effect not demonstrated in clamp studies. What factors might underlie this detrimental effect of meal ingestion on microvascular endothelial function in obese individuals in the present study? One possibility is the levels of postprandial hyperglycemia. Hyperglycemia is widely viewed as major stimulus for the production of reactive oxygen species[24] and previous studies have demonstrated that acute hyperglycemia could induce endothelial dysfunction.[25,26] In the present study, despite their normal fasting glycemia, obese individuals displayed significantly higher postprandial glucose levels compared with lean controls. Since euglycemia is maintained in the studies using the hyperinsulinemic clamp technique[8,10,22] this might explain part of the discrepancy found between the studies. However, since skin microvascular responsiveness was not significantly blunted after ingestion of the glucose load, despite even higher levels of hyperglycemia, other, 'meal-specific', factors may also play a role. Similarly to hyperglycemia, postprandial hypertriglyceridemia has been shown to cause endothelial dysfunction via enhanced oxidant stress.[27,28] Although we did not measure postprandial lipidemia, studies have demonstrated that obesity is associated with higher postprandial triglyceride levels,[29,30] and therefore possible effects of hypertriglyceridemia on endothelial function cannot be excluded. Again, the timing of the microvascular measurement performed could play a role in such effects since triglyceride levels peak within 2-3 h after meal ingestion and larger effects of hypertriglyceridemia on endothelial function can possibly be found after a longer period of triglyceride elevation. Alternatively, our finding of decreased endothelial function with meal ingestion in obese individuals might be explained by the concept of selective insulin resistance. It has been well documented that with insulin resistance there is a selective impairment of insulin's vasodilatory action, whereas its vasoconstrictive effect

remains intact.[31] As a result, insulin can actually cause vasoconstriction in the presence of selective insulin resistance.[32,33] Furthermore, it is possible that postprandial hyperglycemia or hypertriglyceridemia could further acutely accentuate this selective insulin resistance in obese insulin-resistant individuals.

There is convincing evidence that obesity is associated with microvascular dysfunction.[1,34] However, not all studies examining microvascular function in obesity show similar results. Specifically, controversy exists whether microvascular perfusion under resting (unstimulated) conditions is affected in obese individuals. Although reduced capillary density, so-called rarefaction, has been identified in the skeletal muscle of obese Zucker rats[35,36] and obese individuals,[37] most studies have not demonstrated reduced microvascular perfusion in skin[8,38,39] or skeletal muscle[20,22] of obese individuals in the basal state, but have reported reduced endothelial reactivity and capillary recruitment in response to acetylcholine, reactive hyperemia, or insulin.[8,22,23,38] In contrast, some studies have not found a decrease in the response to acetylcholine.[38,40] In the present study, we found no differences in microvascular function between lean and obese individuals in the basal, preprandial, state. We suggest that the discrepancies between various studies might be explained by the different techniques used and by the different characteristics of the populations examined. In the current study, blood pressure (by design) was very similar in both groups, whereas a body mass index difference of 10kg/m^2 (as in the present study) is, on average, associated with 6/4 mmHg (SBP/DBP) higher blood pressure.[41] Indeed, in studies which found impaired microvascular function in the basal state in obesity, blood pressures (even in the normotensive range) were higher in the obese as compared to the lean group.[8,22,38] Taken together, these data raise the possibility that impairment of basal microvascular function in the obese state depends on whether blood pressure is truly normal or, as is most often the case, subtly increased.

In summary, our data demonstrate that neither an oral glucose load nor a mixed meal drink is associated with increases in skin nutritive capillary density or endothelium-(in) dependent vasodilation in either lean or obese individuals. This contrasts with findings of increased nutritive microvascular perfusion during steady-state hyperinsulinemia obtained by an insulin clamp and might indicate that microvascular responses to meal ingestion differ from those induced by the insulin clamp. In particular, this might be related to the amount of and the dynamic nature of the hyperinsulinemia following meal ingestion and/or the complex neuroendocrine responses to feeding. In obese individuals, a mixed meal drink was associated with decreased basal and acetylcholine-stimulated skin perfusion, which is consistent with impaired postprandial microvascular function in obesity. The impaired microvascular function after meal ingestion in obese individuals paralleled blunted insulin-stimulated glucose disposal in these individuals, which is consistent with a role for insulin-stimulated microvascular vasomotion in insulin-mediated glucose uptake in

daily life, and consequently with a role for microvascular dysfunction in the development of insulin resistance in obesity.

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7

General discussion

GENERAL DISCUSSION

In this thesis we focused on the role of microvascular dysfunction, in particular microvascular insulin resistance, as a pathophysiological mechanism in the association between hypertension and metabolic insulin resistance. Clarification of pathophysiological pathways that contribute to microvascular dysfunction might play an important role in the development of therapeutic strategies to prevent and/or treat insulin resistance or type 2 diabetes. To unravel whether overactivity of the renin-angiotensin system (RAS) in the hypertensive state is a possible pathophysiological mechanism underlying microvascular insulin resistance and consequently the development of metabolic insulin resistance, we investigated in **chapters 3 and 4** the effects of Angiotensin II (AngII) and an AngII AT_1-receptor blocker (ARB) on insulin-induced capillary recruitment in both healthy and hypertensive individuals, respectively. In addition, to further explore the role of insulin-induced microvascular actions in glucose metabolism in a physiological (i.e. daily life) setting rather than during an insulin clamp, we investigated the effects of meal and glucose ingestion on the microvasculature in both lean and obese (insulin-resistant) individuals (**chapters 5 and 6**). In the present chapter, the obtained results will be discussed and put into a broader perspective.

Definition of microvascular capacity

Consistent terminology is essential for a detailed discussion of microvascular variables measured in different studies. In the next paragraphs the term *microvascular capacity* is used for the total volume of blood available in the microvasculature for nutrient exchange to tissue. This term encompasses both perfused capillary density (i.e. exchange surface area) and flow (ml/min) per capillary or microvascular unit.

Interaction between angiotensin II and insulin-induced capillary recruitment as a mechanism underlying the association between the RAS and insulin sensitivity

As described in **chapter 1**, several studies indicate that the RAS is involved in the regulation of insulin-mediated glucose uptake and, moreover, that this regulation might be altered in hypertensive (insulin-resistant) individuals compared to healthy individuals. This has led to discussion about the underlying mechanisms for these findings. In **chapters 3 and 4** we investigated whether an interaction between AngII and insulin at the level of the microcirculation underlies, at least in part, this association. We specifically examined the effects of the RAS on *insulin-induced* capillary recruitment, since strong evidence exists that insulin-induced capillary recruitment is crucial for glucose disposal[1,2] and since multiple mechanisms for the RAS to interact with vascular insulin signaling have been identified (as extensively substantiated in **chapter 1**).[3-5] Based on the role of insulin-induced capillary recruitment in glucose disposal[1,2] and based on findings of (nutritive) capillary

recruitment and augmented microvascular blood volume with AngII infusion in rats,[6,7] we specifically hypothesized that AngII, in healthy individuals, would facilitate insulin-induced redistribution of flow towards nutritive capillaries (i.e. nutritive capillary recruitment), as a mechanism underlying the insulin-sensitizing effect of AngII. This might be induced by specific AngII-induced constriction of functional vascular shunts and/or AT_2R-mediated vasodilation of arterioles feeding nutritive capillary beds. Conversely, in hypertensive individuals, where RAS activity might be increased, we hypothesized that AngII may antagonize insulin-induced capillary recruitment via an increased vasoconstrictor response[8] and a direct adverse effect of AngII on insulin PI3K signaling in the endothelium.[3,5,9] Consequently, we supposed that blocking the RAS, rather than infusing AngII, would improve the capillary responses to insulin and, subsequently, insulin-mediated glucose uptake in hypertensive individuals.

In **chapter 3** we confirmed that physiological steady-state hyperinsulinemia increases the number of perfused capillaries in the skin of healthy individuals. Also, we confirmed that acute systemic AngII infusion augments insulin-mediated glucose uptake. However, contrary to our hypothesis, AngII infusion reduced, rather than increased, insulin-mediated capillary recruitment. In **chapter 4** we demonstrated, in line with our hypothesis, that administration of a single dose of irbesartan (AngII AT_1R blocker) indeed increased insulin-induced capillary recruitment in hypertensive individuals. However, no beneficial effect of irbesartan on insulin-mediated glucose uptake was found. Thus, our data suggest that the stimulatory effect of short-term AngII infusion on glucose uptake in healthy individuals is probably not related to increases in capillary recruitment (i.e. exchange surface area) as AngII infusion decreased capillary recruitment during hyperinsulinemia. As described above, rat studies demonstrated AngII-induced capillary recruitment in a hindlimb model and increased microvascular blood volume after systemic AngII infusion in an *in vivo* study.[6,7] However, the latter study also demonstrated increased microvascular blood volume with intravenous injection of the AT_1R blocker losartan.[6] Regardless, we stress that these data should be compared with caution. First, because in our studies the capillary responses to AngII were measured during hyperinsulinemia, whereas in the rat studies the effects of AngII were determined either in the absence of insulin or at fasting plasma insulin levels.[6,7] It is likely that, in our study, a specific interaction between AngII and insulin on microvascular level has occurred, which was absent or less evident in the rat studies.[6,7] Second, because humans are not rats and in contrast to AT_2R-mediated vasodilation in animals, the expression and functional effects of AT_2R in humans have so far not been clearly elucidated.[10,11] Third, because the microvascular variables measured were not exactly the same. Specifically, we examined skin capillary density (exchange surface area), whereas in the in vivo rat study muscle microvascular blood *volume* was measured, which is a measure of both exchange area and flow within the microvasculature.[2,6]

Taken together, the studies described in this thesis are the first that directly investigated the interaction between RAS and insulin in the regulation of insulin-induced capillary recruitment and insulin-mediated glucose uptake in humans. When solely focusing on the effects of AngII on insulin-induced capillary recruitment, we demonstrated that AngII infusion decreased insulin-induced capillary density in healthy individuals, whereas AngII blockade increased insulin-mediated capillary recruitment in hypertensive individuals. We might conclude from these combined data that AngII negatively interferes with insulin-induced capillary recruitment. This is consistent with the reported direct negative effect of AngII on insulin signaling pathways in endothelial cells.[3,4] However, when focusing on insulin-mediated glucose uptake, we found, in both our studies, a dissociation between the microvascular and metabolic effects of AngII. In this regard, it should be taken into account that glucose uptake is not solely defined by delivery, but that other actions of insulin play a role as well. Therefore, it is conceivable that the small changes in insulin-induced capillary recruitment in our studies (~5%) are overruled by other, compensatory or counterregulatory mechanisms important for glucose uptake. Ideally, in future studies both AngII and ARB should be administered in healthy as well as hypertensive individuals to unravel whether microvascular responses and possibly counterregulatory mechanisms differ between these individuals. In addition, further studies on the effects of AngII and ARB on microvascular capacity with and without concomitant insulin infusion are necessary to exclude the possibility that our findings are a general effect on capillary density and not specific for the interaction with insulin.

Postprandial microvascular capacity in lean and obese individuals

Using the euglycemic hyperinsulinemic clamp technique it has been clearly demonstrated that hyperinsulinemia induces skin capillary recruitment, enhances skin endothelium-dependent vasodilation, increases muscle microvascular blood volume, and stimulates intramuscular vasomotion in healthy individuals.[12-20] Moreover, these actions have been shown to be blunted in obesity.[13,18] However, a clamp does not mimic the more complex neuroendocrine response that derives from meal ingestion, such as hyperglycemia, changes in amino acids and gut hormones. Thus, to obtain more information about microvascular responses in a physiological (i.e. daily life) setting, we investigated skin microvascular responses to a glucose load and a mixed meal in lean and obese individuals (**chapter 5 and 6**).

In contrast to findings during an insulin clamp,[12-16,21] we demonstrated no effect of glucose or mixed meal ingestion on perfused skin capillary density or skin endothelium-dependent vasodilation in healthy individuals. However, we did find an increase in skin microvascular vasomotion in healthy individuals after both ingestion of the glucose load and the mixed meal, with a more pronounced increase in vasomotion after the meal

(**chapter 5**). The latter might be secondary to the higher plasma levels of insulin after the meal compared to the glucose load. However, given the complex neuroendocrine responses to feeding[22] other factors cannot be excluded. In addition to our finding of increased skin microvascular vasomotion after mixed meal ingestion, several other studies, using contrast-enhanced ultrasound, demonstrated that ingestion of a meal increases microvascular blood volume in healthy volunteers.[20,23,24] Fewer data are available on the effect of glucose ingestion and findings are less consistent. Some investigators demonstrated enhanced endothelial reactivity in the skin after ingestion of a glucose load,[25] whereas others reported no effect of glucose ingestion on muscle microvascular blood volume[26] or endothelial function,[27,28] or reduced endothelial function after a glucose load.[29,30] However, several outcomes of these investigations may have been confounded by methodological issues, such as the use of different protocols for the measurement of microvascular blood volume and endothelial reactivity compared to meal studies.[25,26]

Concerning obese individuals, we demonstrated that, like findings during an insulin clamp, obese compared to lean individuals are characterized by defects in skin microvascular function in the postprandial state; i.e. obese individuals showed blunted meal-induced stimulation of vasomotion and reduced endothelium-dependent vasoreactivity after meal ingestion. Compared to preprandial values, endothelial reactivity actually diminished in response to mixed meal ingestion in these individuals. Notably, impairment of these measures of postprandial microvascular function paralleled elevated postprandial plasma glucose levels in these individuals, which suggests a role for postprandial microvascular function in insulin-stimulated glucose disposal. However, the small sample size in this study did not allow for a direct correlation between postprandial microvascular function and insulin-mediated glucose uptake. Therefore, other factors (e.g. impaired intramyocellular glucose phosphorylation or impaired suppression of endogenous glucose production after eating) could not be excluded.

Taken together, based on our vasomotion data and collective evidence from other studies,[20,23,24] we reason that mixed meal ingestion indeed stimulates microvascular capacity in healthy individuals. Moreover, we, and others, provided evidence that this response is blunted in obesity.[23] However, the fact that we could not demonstrate these effects consistently with all our techniques, whereas we have shown microvascular responses with different techniques during the clamp (e.g. capillary recruitment and increased endothelial reactivity),[12-16,21] strongly indicates, but does not prove, that microvascular responses during postprandial hyperinsulinemia differ from those during the clamp. In particular, this might be related to the amount of and the dynamic nature of the hyperinsulinemia following meal ingestion and/or the complex neuroendocrine responses to feeding. We speculate that, as a result of a possibly less robust, more dynamic microvascular response, the timing of the microvascular measurements performed and the technique used might be decisive

in detecting, or not detecting a microvascular response to meal feeding. Indeed, not all techniques measure exactly the same component of microvascular capacity (i.e. exchange surface area and/or flow) and different aspects may be differentially sensitive/responsive to insulin infusion or meal ingestion. However, the microvascular responses to mixed meal feeding and insulin infusion in terms of their temporal and quantitative aspects have never been directly compared. Therefore, direct comparisons of skin and intramuscular methods as well as direct comparisons between clamp and postprandial responses are desirable. In addition, more research is necessary to elucidate whether it is indeed the endogenous insulin production that provokes a microvascular response or whether other aspects of the complex neuroendocrine response after feeding underlie this response.

The role of insulin's microvascular actions in regulating glucose uptake – summary

Based on extensive evidence for a stimulatory effect of insulin on the microcirculation[1,2] and based on findings of impaired insulin-induced microvascular actions in the obese insulin-resistant state,[13,15,16,18,31-37] decreased glucose uptake after inhibition of insulin's microvascular actions[14,20,38-45] and improved glucose uptake after restoration of insulin-induced capillary recruitment[32] we used a simple model of insulin's actions for the design and conduct of our studies. Namely, that the regulation of capillary recruitment by insulin is a key component of its action and, when impaired, is a major contributor to metabolic insulin resistance. Thus, based on this model, we expected AngII or ARB-induced changes in insulin-induced capillary recruitment to directly parallel changes in glucose disposal. However, both in **chapter 3 and 4** we found dissociation between the microvascular and metabolic effects of AngII. This suggests that our model is too simplistic. Indeed, besides delivery, other actions of insulin are involved in the regulation of glucose disposal.

Muscle glucose uptake defined

The physiological regulation of muscle glucose uptake requires that glucose travels from the blood to the interstitium to the intracellular space to be phosphorylated to glucose 6-phosphate.[46] Therefore, sites involved in the regulation of glucose uptake can be defined by a three-step process consisting of: (1) delivery of insulin and glucose to the tissue (via skeletal muscle capillaries), (2) transport of insulin and glucose across the endothelial barrier, and (3) uptake of glucose into muscle by translocation of the insulin-responsive glucose transporter GLUT-4.[1,46-49] Insulin is involved in the regulation of these three steps – delivery, transendothelial transport (TET), and uptake into muscle cells – and consequently alterations in any of these actions (due to pharmacological interventions or disease) might change glucose uptake. The coupling of these processes involved in the influx of glucose is illustrated in Figure 7.1 and in the next paragraph we delineate these steps in more detail.

The first action of insulin is to stimulate delivery. As described in **chapter 1**, there is

extensive evidence for a stimulatory effect of insulin on the microcirculation.[1,2] This action is often described as insulin-induced capillary recruitment (i.e. recruitment of previously non-perfused capillaries) even though the majority of research papers invoking capillary recruitment have not visualized the capillary bed.[50] Contrarily, some authors debate the occurrence of capillary recruitment and suggest that insulin augments blood flow within already perfused capillaries.[50,51] We suggest that insulin's actions on the microvasculature to increase delivery might comprise different components. (1) Insulin might induce a more continuous flow of red blood cells within capillaries (prolonged "open time" of supplying arterioles). (2) Insulin might increase functionally perfused capillary volume within already perfused capillaries, possibly via thinning of the glycocalyx,[52] and (3) insulin might recruit previously unperfused capillaries. How insulin exactly stimulates microvascular perfusion might have consequences for detecting changes in microvascular perfusion using different techniques. Besides delivery, insulin TET is a second potential site for regulating glucose uptake.[1] Recent in vivo and in vitro studies provide evidence that insulin traverses the vascular endothelium via a transcellular, receptor-mediated pathway in which PI3K is involved, and emerging data indicate that insulin acts on the endothelium to facilitate its own TET.[1,17,53-57] In line with a role for insulin in its own TET, inflammatory factors and oxidative stress have been shown to diminish insulin uptake by cultured bovine aortic endothelial cells.[56] Moreover, TET has been shown to be substantially delayed in insulin-resistant rats.[58] The third action of insulin is to stimulate glucose uptake in muscle cells via translocation of the insulin-responsive transporter GLUT-4.[59] This requires PI3K-dependent signaling pathways that bear striking similarities with insulin signaling pathways in the endothelium.[48] It has been shown that inappropriate fat accumulation in muscle cells or the release of inflammatory cytokines by fat cells might directly affect muscle myocyte insulin signaling.[49,60-62] Thus it must be kept in mind that besides alterations in insulin-induced microvascular capacity also alterations at the level of insulin's TET or GLUT-4 translocation could account for changes in insulin-mediated glucose disposal.

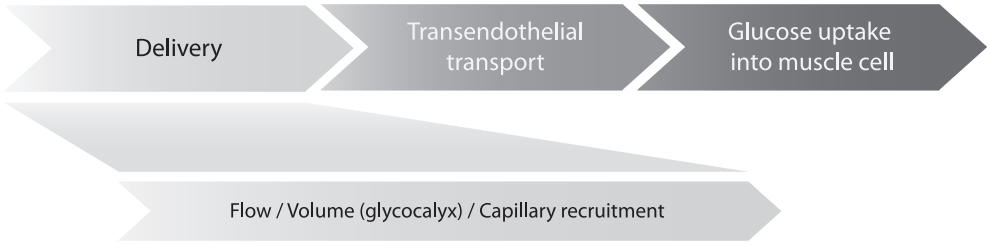

Figure 7.1. The coupling of actions involved in the regulation of insulin-mediated glucose uptake; delivery, transendothelial transport (TET), and uptake of glucose into muscle cells. Insulin's action to increase delivery (i.e. microvascular capacity) might encompass increases in flow, decreases in glycocalyx volume, and capillary recruitment.

Examining the rate-limiting defect for muscle glucose uptake

The aforementioned implies that insulin's actions on TET and GLUT-4 also must be taken into account in studies examining the role for insulin's microvascular actions in regulating glucose uptake (i.e. to identify whether impaired delivery is rate-limiting). However, it might be difficult to distinguish between these different steps of insulin action as most cases of insulin resistance are due to a combination of factors acting to create partial resistance in some, but not necessarily all pathways of insulin action.[63] Thus, some pathways remain sensitive to insulin, and the degree to which a particular pathway becomes resistant may vary from tissue to tissue and between different disease states, depending on the underlying mechanisms. For example, impaired insulin action in the vasculature might develop before dysfunction in skeletal muscle[64,65] as a result of locally produced adipokines from perivascular fat depots that directly and selectively inhibit insulin PI3K signaling in the endothelium.[66,67] However, due to, for example, systemic elevations in FFAs and tumor necrosis factor-α (TNFα)[49,60,68-70] impairments in insulin signaling in endothelial and muscle cells may also develop concomitantly. Therefore, dependent on whether insulin resistance is displayed primarily on the basis of defective delivery or predominantly on the basis of impaired muscle cell insulin action, one would expect strategies to alter microvascular capacity to be more or less effective in improving glucose uptake.[71,72] Possibly, this principle might explain our findings in **chapter 4**. Like TNFα and FFAs,[14,20,68-70] AngII has been shown to affect insulin signaling in skeletal muscle cells as well as endothelial cells.[3-5,35,73-75] Chronic RAS activation, as found in hypertension, could thus possibly impair insulin-mediated GLUT-4 translocation in muscle cells. As a result, it might be likely that the small increase in insulin-induced capillary recruitment (i.e. 4%) as found with a single dose of irbesartan in our study could not result in an increase in overall glucose disposal due to defects in insulin action downstream the endothelium. Noticeably, here, the dosage of the treatment might play a role, since it might be possible that detrimental actions of AngII were mainly blocked in the microcirculation and not yet within skeletal muscle after a single dose of irbesartan. This suggestion is in line with findings of increased intramyocellular insulin-mediated GLUT-4 translocation and GLUT-4 expression after chronic ARB treatment in the obese Zucker rat, and the lack or attenuation thereof after acute ARB treatment.[76,77] It is likely that, due to improvements in intramyocellular insulin action with chronic ARB treatment, perfusion would become relatively more rate-limiting as a result of which improvements in delivery could possibly contribute to the complete normalization of insulin-mediated glucose uptake. However, this needs to be further investigated.

Our data seem to support the concept of a more complex model in which several actions of insulin are involved and in which the contribution of these actions might differ between different pathophysiological states. We suggest that this model should be taken into account in all studies examining insulin's microvascular actions. More precisely,

investigators examining the contribution of insulin's microvascular actions to glucose uptake by means of modulating of insulin's microvascular actions with pharmacological or vasoactive agents must be sure that these agents do not have flow-independent effects on glucose uptake. However, for most of the agents used so far (e.g. FFAs, TNFα, and even L-NMMA)[14,20,38-45] this cannot be completely excluded.[49,60-62,69,78] As a result, concomitant impairments in insulin PI3K signaling in endothelial as well as muscle cells might underlie the pharmacological induced reduction in glucose disposal. Besides, in studies demonstrating impaired insulin-mediated capillary recruitment in the insulin-resistant state,[13,15,16,18,31-37] it is very likely that in the insulin-resistant state simultaneous impairments in both delivery, TET, and GLUT-4 translocation might also play a role.

Main conclusions and future perspectives

In this thesis we focused on the role for insulin's microvascular actions in regulating glucose uptake. We investigated the effects of pharmacological modulation (i.e. AngII and ARB) of insulin-induced capillary recruitment on glucose uptake in healthy and hypertensive individuals and the effects of mixed meal and glucose ingestion on microvascular responses in insulin-sensitive lean and insulin-resistant obese individuals. We demonstrated that AngII interferes with insulin-induced capillary recruitment. We also demonstrated that the microvasculature responds to daily life stimuli (i.e. meal and glucose ingestion) and that this response is blunted in obesity. Yet, in our studies we did not find a direct parallel between (changes in) insulin-induced microvascular function and (changes in) insulin-mediated glucose uptake. We conclude that this might be due to the fact that glucose uptake is not dominated by a single step (i.e. capillary surface area), but that glucose uptake is regulated by a close coupling of different actions, i.e. delivery, TET, and GLUT-4 translocation. These actions are important sites of control and defects in each of these actions can form barriers to muscle glucose uptake. However, the pathogenesis of insulin resistance is complicated and it remains to be determined whether these actions are truly interdependent and if so, what the relative roles of vascular versus intracellular events in total insulin action are in different manifestations of insulin resistance (i.e. hypertension, obesity-induced insulin resistance, genetically based insulin resistance, or diabetes). Importantly, this more complex model of insulin action might have implications for design of therapeutic strategies aimed at the prevention or reduction of insulin resistance and/or diabetes since interventions aimed to improve delivery might be effective in the prevention of metabolic insulin resistance in a very early stage of obesity and/or hypertension (i.e. when microvascular insulin resistance and dysfunction potentially primarily manifest without metabolic insulin resistance), whereas drugs that could potentially correct both vascular and metabolic defects might be more effective to improve glucose homeostasis in a later stage when insulin resistance and diabetes have become entrenched.

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Samenvatting

SAMENVATTING

Het metabool syndroom is een clustering van risicofactoren, zoals overgewicht/obesitas, verhoogd cholesterol, hoge bloeddruk (hypertensie) en metabole insulineresistentie (verminderde insulineafhankelijke glucoseopname), die in combinatie een sterk verhoogd risico geven op het ontwikkelen van hart- en vaatziekten en type 2-diabetes. De prevalentie van het metabool syndroom is hoog en varieert afhankelijk van leeftijd en geslacht, tussen de 10 en 40%. De verwachting is dat de prevalentie de komende jaren verder zal toenemen door de te verwachte stijging in het aantal mensen met overgewicht/obesitas. Inzicht in de mechanismen die ten grondslag liggen aan deze clustering van risicofactoren is dus belangrijk om interventiestrategieën te kunnen ontwikkelen ter preventie of behandeling van hart- en vaatziekten en type 2-diabetes. In dit proefschrift veronderstellen we dat een verminderde werking van de kleine bloedvaten (de microcirculatie) een belangrijke rol speelt in het ontstaan van het metabool syndroom (**hoofdstuk 1**).

De microcirculatie bestaat uit alle vaten kleiner dan 150 µm, dit zijn de arteriolen, capillairen en venulen. De microcirculatie reguleert de aanvoer van zuurstof, hormonen en nutriënten (zoals insuline en glucose) naar de weefsels. Daarnaast reguleert de microcirculatie de bloeddruk via veranderingen in de perifere vaatweerstand. Het hormoon insuline, dat de bloedsuikerspiegel regelt, speelt hierbij een belangrijke rol. Insuline zorgt namelijk voor vaatverwijding via stikstofoxide (NO) productie in het endotheel. Insuline stimuleert de productie van NO in endotheelcellen door achtereenvolgens PI3-kinase en proteïnekinase B te activeren, hetgeen leidt tot activering van stikstofoxidesynthase. Interessant is dat dit insulinesignaaltransductiesysteem (PI3-kinasecomplex) eveneens van belang is voor glucoseopname in de skeletspier (GLUT-4 translocatie). Insuline veroorzaakt verwijding van de weerstandsvaten. Dit resulteert in een toename van de totale spierdoorbloeding. Daarnaast heeft insuline ook een verwijdend effect op de precapillaire arteriolen, met een toename van het aantal doorbloede capillairen (m.a.w. capillaire rekrutering) tot gevolg. Studies hebben aangetoond dat de insulineafhankelijke verwijding van de weerstandsvaten nauw verband houdt met de perifere weerstand terwijl capillaire rekrutering belangrijk is voor de aanvoer van insuline en glucose en daarmee de opname van glucose in spierweefsel. Verder zijn er aanwijzingen dat patiënten met obesitas en hypertensie zich kenmerken door een afgenomen insulineafhankelijke microvasculaire doorbloeding (microvasculaire insuline resistentie) en verminderde glucoseopname in spierweefsel. Verbetering van microvasculaire insulineresistentie is daarom mogelijk een belangrijk therapeutisch doel ter voorkoming van het ontstaan van metabole insulineresistentie in hypertensieve patiënten.

Met de in dit proefschrift beschreven onderzoeken hebben wij getracht om meer inzicht te krijgen in de rol van insulineafhankelijke microvasculaire effecten in de glucoseopname.

Wij wilden ondermeer achterhalen of verhoogde activiteit van het renine-angiotensine systeem (RAS) een mogelijk pathofysiologisch mechanisme is voor het ontstaan van microvasculaire en daarmee metabole insulineresistentie in hypertensie. **Hoofdstuk 2** geeft een beschrijving van de verschillende technieken die in dit proefschrift gebruikt zijn voor het meten van microvasculaire functie. Tevens worden de verschillende technieken voor het meten van insulinegevoeligheid besproken, onder andere de hyperinsulinemische euglycemische clamp. Tijdens een hyperinsulinemische clamp krijgen personen een intraveneuze infusie met insuline. Als gevolg hiervan stijgt de insulineconcentratie en door deze 'hyperinsulinemie' zal de bloedglucoseconcentratie dalen. Om een normale bloedglucose te handhaven (euglycemie) wordt een tweede oplossing met glucose geïnfundeerd. De hoeveelheid glucose die geïnfundeerd moet worden om euglycemie te handhaven is een maat voor de insulinegevoeligheid.

Effecten van angiotensine II op insulineafhankelijke capillaire rekrutering als onderliggend mechanisme voor de relatie tussen het RAS en insulinegevoeligheid

Er is een aanzienlijke hoeveelheid bewijs dat het RAS een rol speelt in het reguleren van glucoseopname (i.e. insulinegevoeligheid). Studies hebben aangetoond dat het blokkeren van het RAS (blokkeren van de aanmaak of de effecten van angiotensine II (AngII)) in hypertensieve personen tot een toename in insulineafhankelijke glucoseopname leidt. Echter, studies hebben eveneens aangetoond dat niet het blokkeren, maar juist het toedienen van AngII in gezonde personen tot een toename in insulineafhankelijke glucoseopname leidt. De relatie tussen het RAS en glucoseopname lijkt dus veranderd in hypertensieve personen, mogelijk veroorzaakt door chronische activatie van het RAS in hypertensie. Omdat insulineafhankelijke capillaire rekrutering kan bijdragen aan de opname van glucose en omdat er duidelijke aanwijzingen zijn dat er een complexe interactie bestaat tussen AngII en het insulinesignaaltransductiesysteem voor de productie van NO in het endotheel, hebben wij in **hoofdstuk 3 en 4** onderzocht of er via het RAS veranderingen in insulineafhankelijke capillaire rekrutering kunnen optreden om zo tot veranderingen in glucoseopname te kunnen leiden. In **hoofdstuk 3** laten we zien dat intraveneuze infusie van insuline in gezonde personen zoals verwacht tot een toename in het aantal doorbloedde capillairen in de huid leidt. Net als in eerdere studies laten we ook zien dat AngII infusie tijdens een hyperinsulinemische euglycemische clamp tot een toename in insulineafhankelijke glucoseopname leidt. Echter deze stijging gaat niet gepaard met een stijging, maar met een daling in insulineafhankelijke capillaire rekrutering. In **hoofdstuk 4** laten wij zien dat de éénmalige inname van een dosis irbesartan (een AngII AT_1-receptorblokker (ARB)) tijdens een hyperinsulinemische euglycemische clamp in hypertensieve personen zoals verwacht tot een toename in insulineafhankelijke capillaire

rekrutering leidt. Echter deze éénmalige dosis ARB gaf geen significante effecten op de insulinegevoeligheid.

Dus wanneer we ons enkel richten op de effecten van AngII op de microcirculatie, blijkt dat AngII toediening in gezonde personen de insulineafhankelijke capillaire rekrutering doet afnemen, terwijl AngII blokkade in hypertensieve personen de insulineafhankelijke capillaire rekrutering doet toenemen. Uit deze gecombineerde data concluderen wij dat AngII een negatief effect heeft op insulineafhankelijke vaatverwijding. Dit is consistent met bevindingen in dierstudies waarin een direct negatief effect van AngII op het insulinesignaaltransductiesysteem in endotheelcellen gevonden is. In beide groepen vinden we echter wel een dissociatie tussen de microvasculaire en metabole effecten van AngII; AngII infusie resulteerde in een afname in insulineafhankelijke capillaire rekrutering en een toename in insulineafhankelijke glucoseopname in gezonde personen; AngII blokkade resulteerde in een toename in insulineafhankelijke capillaire rekrutering zonder verandering in glucoseopname in hypertensieve personen. Dit suggereert dat de kleine veranderingen (~5%) in insulineafhankelijke capillaire rekrutering in beide studies overstemd zijn door andere, compensatoire of tegenwerkende mechanismen die van belang zijn voor insulineafhankelijke glucoseopname.

Postprandiale microvasculaire functie

Bewijs voor een rol van insuline in het reguleren van microvasculaire doorbloeding is voornamelijk geleverd in studies waarin gebruik gemaakt is van de hyperinsulinemische euglycemische clamp techniek (m.a.w. langdurige intraveneuze toediening van insuline). Deze studies hebben aangetoond dat hyperinsulinemie in gezonde slanke personen een toename van het aantal doorbloede capillairen en een toename in endotheelafhankelijke vaatverwijding in de huid tot gevolg heeft. In spierweefsel van slanke personen is een toename in vasomotie (ritmische variatie in spierdoorbloeding) en microvasculair bloedvolume gevonden tijdens de hyperinsulinemische clamp. Daarnaast is met behulp van de hyperinsulinemische clamp aangetoond dat deze microvasculaire acties van insuline gestoord zijn in personen met obesitas. Echter een hyperinsulinemische clamp is een kunstmatige stimulus die niet geheel overeenkomt met een situatie zoals deze zich in het dagelijks leven voordoet. Om meer inzicht te krijgen in de microvasculaire effecten van insuline in een fysiologische situatie hebben we in **hoofdstuk 5 en 6** de effecten van een maaltijddrank en een glucosedrank op de microcirculatie in slanke en obese personen onderzocht (m.a.w. de postprandiale microvasculaire functie).

In tegenstelling tot bevindingen tijdens een hyperinsulinemische clamp vonden wij na de inname van de glucose- of maaltijddrank in gezonde slanke personen geen toename in endotheelafhankelijke vaatverwijding of het aantal doorbloede capillairen in de huid. Echter na de inname van beide dranken werd in slanke personen een toename

in microvasculaire vasomotie in de huid gevonden, met een sterker effect van de maaltijddrank. Dit laatste zou gerelateerd kunnen zijn aan de hogere insulineconcentraties in het bloed na de maaltijddrank in vergelijking met de glucosedrank. In vergelijking tot slanke personen werden obese personen in de postprandiale toestand gekenmerkt door een verminderde functie van de microcirculatie. Obese personen lieten namelijk geen maaltijdgeïnduceerde toename in microvasculaire vasomotie zien en toonden een verminderde endotheelafhankelijke dilatatie na inname van de maaltijd. Naast een gestoorde postprandiale microvasculaire respons werden obese personen gekenmerkt door langdurig verhoogde postprandiale glucoseconcentraties in het bloed (waarschijnlijk veroorzaakt door een gestoorde glucoseopname).

Aan de hand van de vasomotiedata in dit proefschrift en aan de hand van bevindingen van toegenomen spierdoorbloeding na een maaltijd in andere studies suggereren wij dat in gezonde personen inname van een maaltijd inderdaad een toename in microvasculaire doorbloeding tot gevolg heeft. Bovendien hebben we via de studies beschreven in **hoofdstuk 5 en 6** aangetoond dat deze postprandiale microvasculaire respons gestoord is in obese personen. Echter, het feit dat we na een maaltijd niet met alle technieken veranderingen in microvasculaire functie konden aantonen, terwijl dit tijdens een hyperinsulinemische clamp wel aangetoond is, wijst er sterk op dat de microvasculaire respons tijdens postprandiale hyperinsulinemie anders is dan tijdens een hyperinsulinemische clamp. Dit zou verband kunnen houden met de lagere plasma insulineconcentraties die bereikt worden met een maaltijd in vergelijking met een hyperinsulinemische clamp, maar ook met het feit dat een maaltijd voor een korte piek in plasmaconcentraties zorgt terwijl de insuline spiegels tijdens een hyperinsulinemische clamp langdurig hoog worden gehouden. Aanvullend onderzoek is nodig om de microvasculaire effecten tijdens een maaltijd beter in kaart te brengen en deze te vergelijken met de respons na een hyperinsulinemische clamp. Daarnaast is het van belang om te achterhalen of inderdaad endogeen geproduceerd insuline (i.e. insuline geproduceerd door de alvleesklier in reactie op de verhoogde glucoseconcentraties) verantwoordelijk is voor de microvasculaire effecten na een maaltijd, of dat andere aspecten van de complexe neuronendocriene reflex na een maaltijd hier aan ten grondslag liggen.

Algemene conclusie

Hoofdstuk 7 is een algemene beschouwing van de bevindingen in dit proefschrift. Hierin staat onder meer beschreven dat aan de hand van de studies in dit proefschrift geconcludeerd kan worden dat insulineafhankelijke microvasculaire functie niet altijd direct één op één verband houdt met de metabole effecten van insuline. Dit betekent allerminst dat de microvasculaire effecten van insuline onbelangrijk zijn voor glucoseopname, zonder toevoer van insuline en glucose naar de spierweefsels is opname

in spiercellen immers niet mogelijk. Echter in de studies beschreven in dit proefschrift zijn andere factoren naar alle waarschijnlijkheid belangrijkere determinanten voor de opname van glucose. Hierbij valt bijvoorbeeld te denken aan het transport van insuline door het endotheel (van plasma naar interstitium) en opname van glucose in de spiercel (door translocatie van de glucosetransporter GLUT-4). Het is dan ook belangrijk om te realiseren dat insulineresistentie een complexe aandoening is en dat insulineresistentie (in bijvoorbeeld hypertensie of obesitas) vaak veroorzaakt wordt door een combinatie van factoren die gedeeltelijke insulineresistentie in sommige, maar niet noodzakelijkerwijs in alle insulinesignaaltransductiesystemen veroorzaken. Afhankelijk of insulineresistentie primair veroorzaakt wordt door verminderde toevoer van insuline en glucose naar de weefsels, gestoorde insulinesignaaltransductie in het endotheel, of overwegend het gevolg is van een gestoorde actie van insuline in de spiercellen, zou je kunnen verwachten dat therapeutische strategieën gericht op het verbeteren van microvasculaire functie meer of minder effect hebben op het verbeteren van de glucoseopname.

Dankwoord

DANKWOORD

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Curriculum Vitae

CURRICULUM VITAE

Amy Maria Jonk is geboren op 7 september 1982 te Purmerend. Zij groeide op in Volendam, waar zij in 2000 haar VWO diploma behaalde aan het Don Bosco College. In datzelfde jaar startte zij de studie Bewegingswetenschappen aan Vrije Universiteit in Amsterdam. Tijdens deze studie heeft zij onderzoek gedaan naar de ploegenachtervolging in het langebaanschaatsen in Nederland, Noorwegen en Italië. In 2004 won zij de G.J. van Ingen Schenau Promising Young Scientists Award, waarna zij in 2005 een extra wetenschappelijke stage heeft gedaan in San Diego (Californië, Verenigde Staten) op het gebied van hoogtefysiologie. In februari 2006 behaalde zij cum laude het doctoraalexamen, waarna zij direct aansluitend is begonnen als onderzoeksassistent aan de Faculteit der Bewegingswetenschappen van de Vrije Universiteit om in samenwerking met TNO en NOC*NSF onderzoek te doen naar de ontwikkeling van handbikes voor de Paralympische Spelen van Beijing 2008. In juni 2006 startte zij een promotietraject bij de afdeling Interne Geneeskunde van het Maastricht Universitair Medisch Centrum en Cardiovascular Research Institute Maastricht, onder begeleiding van haar promotores Prof. dr. C.D.A. Stehouwer en Prof. dr. de Leeuw en copromotor dr. A.J.H.M. Houben.

Amy Maria Jonk was born on September 7th 1982 in Purmerend, the Netherlands and she was raised in Volendam. In 2000 she graduated her secondary school education (VWO) at the Don Bosco College in Volendam. In the same year she started to study Human Movement Sciences at the Vrije Universiteit in Amsterdam. During her study she performed a scientific internship on the team pursuit in speed skating in The Netherlands, Norway and Italy. In 2004 she received the G.J. van Ingen Schenau Promising Young Scientists Award and conducted an additional scientific internship at the University of California San Diego were she studied exercise performance at high altitude. In February 2006 she obtained her Master's degree cum laude. Subsequently, she started as a research assistant at the Faculty of Human Movement Sciences at the Vrije Universiteit Amsterdam to conduct, in corporation with TNO and NOC*NSF, research on the design of handbikes for the Dutch Paralympic team. In June 2006 she started her PhD at the department of Internal Medicine at the Maastricht University Medical Centre and the Cardiovascular Research Institute Maastricht, under supervision of her promotores Prof. dr. C.D.A. Stehouwer and Prof. dr. de Leeuw, and copromotor dr. A.J.H.M. Houben.

List of publications

FULL PAPERS

Jonk AM, Houben AJ, de Jongh RT, Serné EH, Schaper NC, Stehouwer CD. Microvascular dysfunction in obesity: a potential mechanism in the pathogenesis of obesity-associated insulin resistance and hypertension. *Physiology* 2007;22:252-260.

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Jonk AM, Houben AJ, Schaper NC, de Leeuw PW, Serné EH, Smulders YM, Stehouwer CD. Meal-related increases in microvascular vasomotion are impaired in obese individuals: a potential mechanism in the pathogenesis of obesity-related insulin resistance. *Diabetes Care* 2011; 34 (Supplement 2):S342-S348.

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de Boer MP, Meijer RI, Wijnstok NJ, **Jonk AM**, Houben AJ, Stehouwer CD, Smulders YM, Eringa EC, Serné EH. Microvascular dysfunction: a potential mechanism in the pathogenesis of obesity-associated insulin resistance and hypertension. *Microcirculation* 2011 Aug 28; [Epub ahead of print].

Houben AJ, Eringa EC, **Jonk AM**, Serné EH, Smulders YM, Stehouwer CD. Perivascular fat and the microcirculation: relevance to insulin resistance, diabetes and cardiovascular disease. *Curr Cardiovasc Risk Rep*; accepted.

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List of abbreviations

LIST OF ABBREVIATIONS

1-MX	1-methylxanthine
αMT	α-methylserotonin
ABPM	Ambulatory blood pressure monitoring
ACE	Angiotensin-converting enzyme
Akt	Protein kinase B
AngII	Angiotensin II
ARB	Angiotensin II AT_1R blocker
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
BMI	Body mass index
CCB	Calcium channel blocker
DBP	Diastolic blood pressure
BP	Blood pressure
EDHF	Endothelium-derived hyperpolarization factor
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-regulated kinase-1/2
ET-1	Endothelin-1
FFA	Free fatty acid
GLUT-4	Glucose transporter
HOMA2-IR	Homeostasis model assessment for insulin resistance
HR	Heart rate
IL-6	Interleukin-6
IRS	Insulin receptor substrate
IRS-1	Insulin receptor substrate-1
i.v.	Intravenous
L-NMMA	L-NG-monomethyl Arginine citrate
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
M value	Whole-body glucose uptake
M/I value	Insulin sensitivity
NFκB	Nuclear factor-kappaβ
NO	Nitric oxide
OGTT	Oral glucose tolerance test
PDK-1	Phosphoinositide-dependent kinase-1
PE	Phenylephrine
PGE2	Prostaglandin E2
PGI2	Prostacyclin
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PRH	Postocclusive reactive hyperemia
RAS	Renin-angiotensin system
RIA	Radioimmunoassay
ROS	Reactive oxygen species
SBP	Systolic blood pressure
TET	Transendothelial transport
TNFα	Tumor necrosis factor-α